mice after 8 to 25 passages in either chicken embryos, chicken embryonic tissue cultures, or suckling hamsters.

Viremic chicks rather than viremic horses were used in this study as a source of virus to infect mosquitoes. However, the possibility that *C. tarsalis* could become infected by feeding on the blood of a viremic horse vaccinated with Clone 15 WEE virus seems remote. Kemp and Johnson(3) reported that even those horses injected intramuscularly with large doses of the attenuated WEE virus circulated less than $10^{1.0}$ LD_{50} of virus/0.01 ml of blood, and this low level of viremia would not be expected to infect *C. tarsalis*.

Summary. *Culex tarsalis* can be infected with a non-neuroadapted strain of western equine encephalomyelitis virus and an attenuated variant with low neuropathogenicity derived from this strain. The infective threshold of the attenuated variant for *C. tarsalis* was greater than $10^{3.0}$ suckling mouse intracranial LD_{50} of virus/0.01 ml of blood. The original and attenuated strains multiplied equally in mosquitoes and both were transmitted to chicks by mosquito bite. The mouse pathogenicity or plaque size of the attenuated virus was not altered after a single passage in chicks and mosquitoes. The attenuated variant developed lower levels of viremias in chicks than the original strain.

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Immunofluorescence of Human Adenovirus Type 12 in Various Cell Types.* (30791)

JEANA D. LEVINTHAL, CYRUS AHMAD-ZADEH,† GERALD VAN HOOSIER, JR. AND JOHN J. TRENTIN

Department of Bacteriology and Immunology, Harvard Med. School, Boston, Mass., and Baylor University College of Medicine, Houston, Texas

The discovery in 1962 of the oncogenic properties of human adenovirus type 12 (A-12) in hamsters(1,2,3) and the fact that other human adenoviruses and other heterologous species share this property(4,5,6) has brought new facets to the subject of cell-virus relations resulting in cancer. Some animals with primary tumors develop neutralizing antibodies(7), and animals with primary or grafted A-12 tumors develop complement-fixing antibodies to the type specific C antigen (8). Antibodies detectable by complement fixation, gel diffusion(9) and fluorescent antibody(10) to early antigens associated with the adenovirus multiplication cycle are also made. These findings make a study of the antigens detectable by fluorescent antibody in infected and transformed cells of considerable interest. It has been shown by other workers that a nearly parasynchronous single cycle of viral replication can be initiated in susceptible

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† Institut d'Hygiene, Geneva, Switzerland.

cells(11,12,13) by human adenoviruses, and that this cycle can be studied by immunofluorescence over the course of the infection. We have accordingly made observations on the accumulation and localization of the early and structural viral antigens induced by A-12 in cells capable of complete or abortive cell-virus interaction.

Materials and methods. Virus. Adenovirus type 12 (Huie strain) was propagated in S-3 HeLa cells in arginine enriched Eagle's medium, and a pool titering $10^{2.5}$ $TCID_{50}$/0.1 ml in S-3 HeLa cells was frozen at —70°. (Titers determined on human embryonic kidney cells are approximately 4 logs higher.) This pool was used for preparation of antisera in rabbits against structural viral antigens, for tumor induction in newborn hamsters, and for infection of various tissue cultures under identical conditions.

Culture methods. Primary cultures of trypsin dispersed cells of various origins were seeded in Leighton tubes containing coverslips 11 × 39 mm in size, large enough so that 3 or 4 pieces could be broken from a single coverslip for staining with different sera, or for repeated staining as necessary. Eagle's medium (MEM) in Earle's saline, with 10% heat inactivated (56° for ½ hr) fetal calf serum, glutamine and antibiotics (100 units penicillin, 100 μg streptomycin, and 1 μg neomycin/ml) was used as the growth medium for all primary cultures, and for human malignant cell lines. The same medium with 1 millimolar arginine was used in all viral studies. Media were changed twice a week for human KB, HEp-2, S-3 HeLa, and primary cell cultures. Five hamster tumor lines, one received through the kindness of Dr. William A. Strohl(14), another generously provided by Dr. David Yohn, 2 put into cultivation by Dr. I. Kitamura(15), and one in the senior author's laboratory, were maintained on an enriched medium consisting of MEM with 4 × the usual concentration of amino acids and vitamins, 10% heat inactivated fetal calf serum, 10% NCTC #109, and 1 mg% liver extract concentrate (Sigma), plus antibiotics. Media were completely changed 3 times a week for these lines. Three Wistar rat kidney cell lines and one New Zealand White rabbit kidney cell line transformed *in vitro* by A-12 in our laboratory (16) were similarly maintained.

Virus inoculation, fixation of monolayers. When monolayers of the various cell types were nearly confluent, 0.1 ml of the virus pool was added to each Leighton tube, after feeding with 1 ml of arginine enriched MEM. At intervals after infection, coverslips from virus infected and uninfected control tubes were hooked out, washed in phosphate buffered saline pH 7.4 and fixed in acetone at —70° for 10 minutes. After warming to room temperature, the coverslips were removed, air dried, and stored in screw cap vials at 4° or —70° until stained. Under the latter conditions, antigens were stable for several months.

Preparation and use of antisera in immunofluorescent staining. Viral structural antigens. Viral pellets were prepared by high speed centrifugation of 10 ml of the crude virus pool, resuspended in 1 ml of medium, and injected intravenously in adult rabbits. A second virus pellet mixed with Freund's adjuvant was injected intramuscularly. After 3 weeks, a booster injection was given intravenously, and the animals were bled a week later. Another booster dose was given at this time, and the animals were bled a second time after 6 days.

Globulin from these sera was precipitated with cold, half-saturated ammonium sulfate, labeled with fluorescein isothiocyanate, and chromatographed on DEAE cellulose columns. The fraction eluting at 0.05 M phosphate buffer, pH 6.3 was concentrated until the immunofluorescent staining of acetone-fixed viral antigens in infected cells was clear and brilliant when reacted with the anti-viral globulin overnight, with a small amount of undiluted guinea pig complement. The specificity of the staining was established as usual: structural viral antigens in predictable patterns were seen only in the nuclei of infected cells, and not in controls. These viral antigens were not stained with fluorescein labeled globulins prepared against other antigens such as SV40 virus or polyoma virus. Furthermore, the morphology, distribution, and time sequence of the appearance of these

intranuclear structural viral antigens resembled that reported in other studies of adenoviruses using this method.

Early antigens (tumor antigens) of the adenovirus cycle. Pooled sera from hamsters bearing grafts of A-12 induced tumors were reacted by the indirect Coons technique with infected and control monolayers, followed by staining with a commercial goat anti-hamster globulin (Baltimore Biological Laboratories), chromatographed on DEAE cellulose and used at a concentration of 0.3% protein. The most frequently used pool which stained only early antigens was one titering 1:256 by complement fixation against hamster A-12 tumor antigen, and used at a dilution of 1:4. A second pool containing antibody to both early and structural viral antigens was purified and fluorescein labeled and was used in staining by the direct technique. In the indirect technique, fixed monolayers were warmed to room temperature, washed briefly with phosphate buffered saline, drained, covered with unlabeled serum and incubated, resting on a paper clip, in a petri dish containing a wad of wet paper, at 36° for 40 minutes. After a 5-10-minute wash, with 3 changes of cold phosphate buffered saline, they were drained and incubated a second time, covered with a drop or two of fluorescein labeled goat anti-hamster globulin for 20 minutes at 36°. After a second 5-10-minute wash, they were drained, mounted in 9:1 glycerine-saline, pH 8.5, and rimmed with fingernail polish to prevent drying. Photographs were taken within a few days of staining, using a Polaroid camera mounted on a Zeiss fluorescence microscope.

Results. Antigens of the viral replication cycle. Only in primate cells could the complete cycle, the production of early antigens followed by production of structural viral antigens, be observed. The appearance of the early antigens, demonstrable by staining with serum of tumorous hamsters, followed a similar pattern in these cells, but with great variation as to amount of material produced, and the brilliance of staining. Enders continuous monkey kidney line produced larger amounts, more brilliantly stained than other cells. At about 6 hours after infection, short sharp pointed flecks, often arrow shaped, appeared in a few nuclei, and similar flecks or slender threads, sometimes more faintly stained, in the cytoplasm. These became clearer and more abundant and appeared in more cells by 9 hours. By 24 hours, at which time the structural antigens were also accumulating in the nuclei, early antigens were present as tangled masses of nuclear threads and flecks, while similar cytoplasmic material often aggregated around the nucleus in a shaggy wreath, or in a localized crescent shaped clump. Sometimes it was difficult to tell whether some of this material was in the cell or stuck to the surface of individual cells. Two common artifacts, the strong tendency for monkey and human kidney cells to show nonspecific staining of the entire nucleus, particularly when they lie near the edge of a preparation, and the similar tendency of cells which round up and lie above the general surface of the monolayer to show diffuse, amorphous staining of the entire cell, add to the difficulties of interpretation, because with the progress of the infection, the adenovirus cytopathic effect causes the cells to round up prior to detachment from the glass. Monkey, human fetal fibroblast and kidney cells with early antigen stained at 24 hours are illustrated in Fig. 1.

The accumulation of structural viral antigens, demonstrable by staining with rabbit antiviral serum, was best observed in human fetal kidney cells, the first appearance of this material coming at about 14 hours after infection, in the form of bright dots just within the nuclear membrane. The normal faint nuclear autofluorescence was diminished at this stage. Ring forms with what appeared to be satellite dots around and within the rings were seen during the 16- to 20-hour period, while other nuclei became filled with antigen in the form of coarse granules. A central granular mass developed during the 26- to 30-hour period, usually surrounded by a bright homogeneous ring of antigen, and connected with it by fine strands across an intervening unstained space. The peripheral ring in contrast with the central mass was not stained with acridine orange (Fig. 2), and therefore does not contain a detectable component of

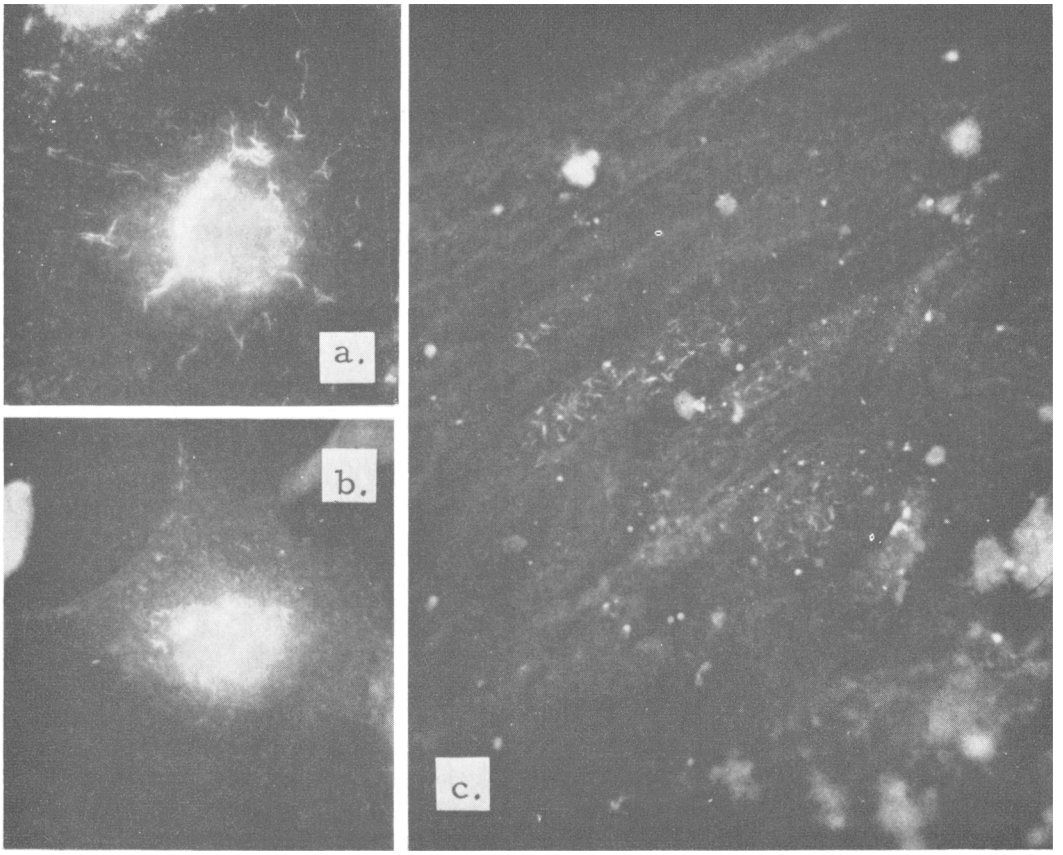

FIG. 1. Demonstration by immunofluorescence with serum of tumorous hamsters, of early antigens of A-12 cycle in infected Enders monkey kidney (a), human fetal kidney (b) and human fetal fibroblast cells (c) stained at 24 hr. Larger, more brilliant aggregates in the monkey cells. Tendency for both monkey and human fetal kidney nuclei to stain nonspecifically, in addition to specific staining of nuclear flecks and threads. Human fibroblasts, well spread in monolayer, show mainly specific staining of nuclear and cytoplasmic antigen (Mag. × 400).

nucleic acid. The central mass was at first relatively unstained compared with the surrounding smooth ring, but later became equally brilliant. The smooth material, usually seen as a peripheral ring was sometimes distributed in irregular plaques and bands in the interior of the nucleus where it surrounded smaller granular masses. The peripheral ring finally retracted in a denticulate fashion from the nuclear membrane leaving another unstained space. During the 30- to 48-hour period, the nucleus became swollen, the cytoplasm rounded up in the typical "adenovirus type CPE" and the cells became detached from the glass. At this stage the nuclear membrane often lost its integrity, and structural viral antigens began to diffuse into the cytoplasm. This sequence is illustrated in Fig. 3.

As noted by other workers(12), the mitotic rate in infected cells drops after the first 12 hours, but the cycle of infection can continue in daughter cells that have divided prior to this time, as one frequently sees paired cells in which the structural viral antigens are accumulating at exactly the same rate, and in the same pattern, although these cells are usually smaller than single infected cells. Two such pairs are illustrated in Fig. 3.

Antigen production in infected cells of various origins. The results of a survey of antigen production after infection with a standard inoculum of A-12 are summarized in Table I.

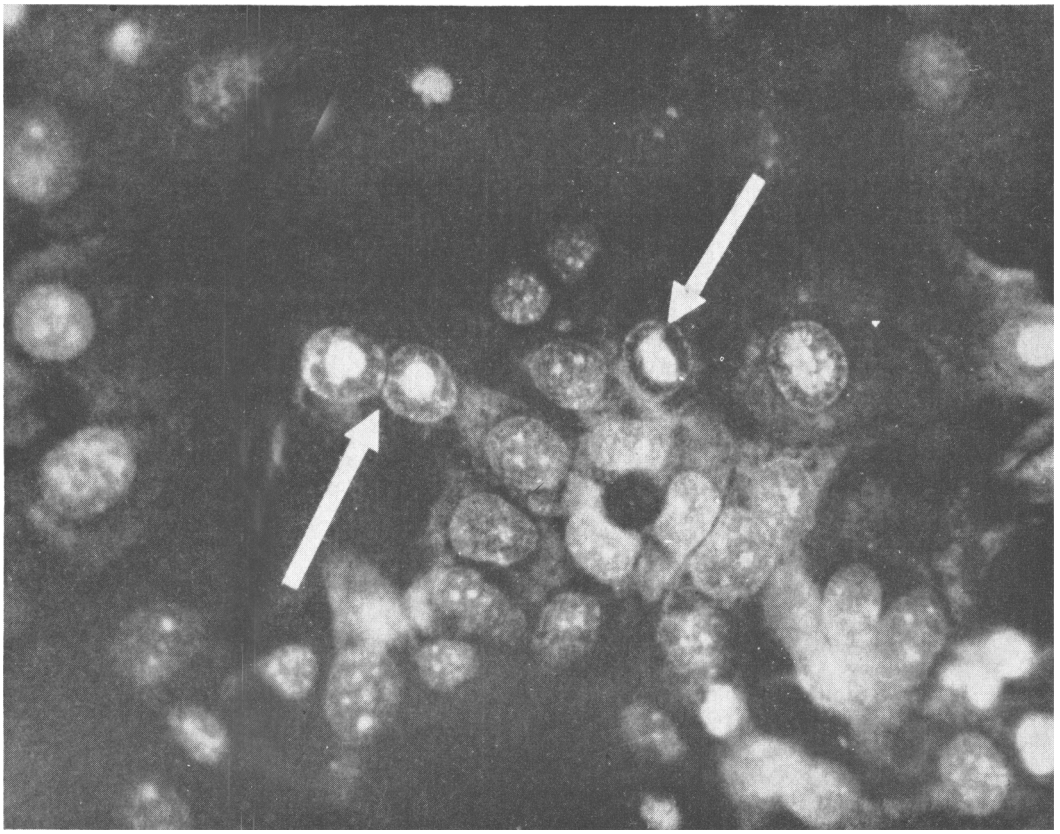

FIG. 2. HEp-2 cells infected with A-12 and stained at 40 hr with acridine orange, which demonstrates the central nuclear inclusion, but not the peripheral nuclear ring of antigen demonstrable by immunofluorescence, (see Fig. 3) (Mag. × 400).

Of all cell types studied, human fetal kidney cells were the most susceptible to undergoing the complete virus-productive, cell-destructive relation, as judged by the proportion of cells showing large amounts of brilliantly stained nuclear viral structural antigens, accompanied by typical "adenovirus type CPE," the cultures being completely destroyed in 3 or 4 days. Of the 3 human malignant lines studied, KB cells most resembled the human fetal kidney, in the high proportion of cells containing brilliantly stained structural viral antigens at 40 hours; HEp-2 cells were almost as effective in replicating structural viral antigens, but in S-3 HeLa cells and in the continuous line amnion cells, their appearance was diffuse, ill defined and far less brilliant, although the cultures developed the adenovirus type CPE very rapidly. Human fetal fibroblasts appeared less susceptible to development of a parasynchronous infection, as only about 30% of these cells contained structural viral antigens at 40 hours, with considerable variation depending upon the source of the fibroblasts. Some cultures were rapidly destroyed by interaction with virus, while in others, so small a proportion of cells contained early or structural antigens at any one time that destruction and proliferation of cells continued in a precarious balance, and some cultures were maintained in this state for 80 days. No transformation appeared however.

As previously mentioned, early antigens were demonstrable in large amounts in nearly every cell of the Enders continuous monkey kidney line, but only a small proportion of these cells continued further with the cycle to produce structural antigens. However, the typical adenovirus cytopathic effect developed

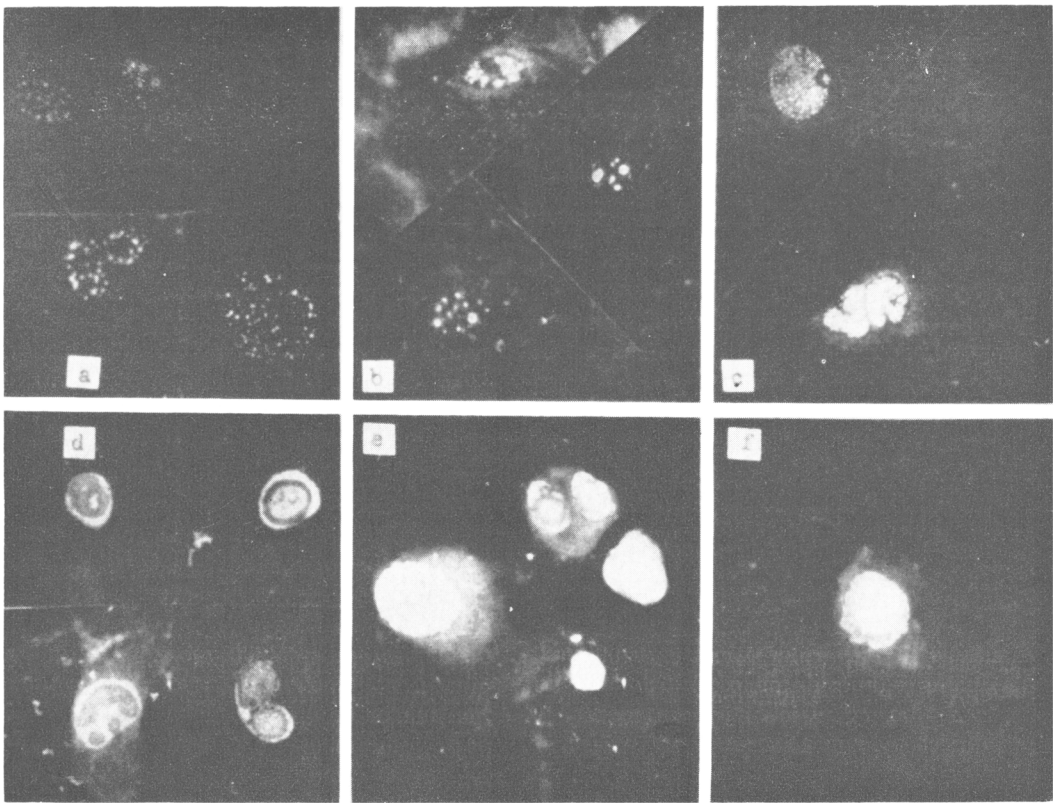

FIG. 3. Sequential accumulation, in the nuclei of human fetal kidney cells infected with A-12, of structural viral antigens demonstrable by immunofluorescence, with rabbit antiviral serum. a. 14 hr; b. and c. 16 to 20 hr; d. and e. 26 to 30 hr; f. 30 to 48 hr (Mag. × 400).

with great rapidity in these cells, and the cultures were destroyed in 3 or 4 days after infection.

Among the 6 types of non-primate cells examined, 4 produced early antigens in a high proportion of cells, and although the cycle went no further than this a moderate cytopathic effect appeared in all. Rat, rabbit, hamster and mouse cells containing early antigens are illustrated in Fig. 4.

When stained in the interval between 48 hours and 10 days, the cells rounding up and leaving the glass contained aggregates of antigen, still staining brilliantly, while neighboring cells that appeared healthy demonstrated no antigen. Chick fibroblast cultures had only a rare cell with demonstrable antigen, and these cells rounded up and were lost from the monolayer; because they were so few, no reaction was visible upon examining the monolayer prior to fixation and staining. Similarly, the Earle L cell showed no CPE after infection with A-12, and no demonstrable early antigen was found in any cell.

Of the 5 A-12 induced hamster tumor lines in culture examined, all showed a scanty production of nuclear and cytoplasmic threads and flecks in the majority of cells, which although clearly visible in the UV microscope were frequently difficult to photograph. Different tumor lines consistently produced more or less of these antigens, but all showed a greatly increased production demonstrable in nearly every cell after superinfection with A-12. Cells with this increased amount of early antigen gradually disappeared from the monolayer over the course of a few days, and the tumor line resumed its previous rate of antigen production. A similar situation was found with a rabbit kidney line transformed *in vitro* by A-12. However, 3 lines of Wistar rat kidney transformed *in vitro* by A-12 did

TABLE I. Production of Antigens of Human Adenovirus Type 12 Cycle in Infected and Transformed Cells as Detected by Immunofluorescent Staining.

		Early antigens		Estimated % cells +	Structural antigens	Estimated % cells +
		Nucl.	Cyto.		Nucl.	
Primate cells	Prim. hum. fet. kidney	+++*	++	90	++++	90
	Human newborn kidney	+++	++	90	++++	90
	Human 3 month kidney	+++	++	90	++++	90
	Prim. hum. fet. fibro. (skin-muscle, lung)	+++	++	30	++++	30
	KB	+++	++	80	++++	80
	HEp-2	+++	++	80	+++	80
	S-3 HeLa	+++	++	80	++	80
	Continuous line amnion WS	+++	++	80	++	80
	Enders monkey kidney	++++	++++	90	+++	1–3
Non-primate cells	Prim. rabbit kidney (New Zealand white)	+++	+++	50–80	—	0
	Prim. hamster kidney (Syrian golden)	+++	+++	50–80	—	0
	Prim. rat kidney (Wistar)	++	++	50–80	—	0
	Prim. mouse kidney (3H/F/Bi/Gs)	++	++	50–80	—	0
	Prim. chick fibroblast	+	+	.1	—	0
	Earle's L cell (mouse fibroblast)	—	—	0	—	0
A-12 transformed cells and tumors in culture	5 hamster tumor lines	+	+	80–100	—	0
	Superinfected A-12	++	++	100		
	3 Wistar rat kidney lines	—	—	0	—	0
	Superinfected A-12	++	++	100		
	1 rabbit kidney line (New Zealand White)	+	+	80–100	—	0
	Superinfected A-12	++	++	100	—	0

* The rating + to ++++ is an arbitrary estimate of the brilliance of staining and amount of material stained.

not contain demonstrable early antigen, even though these cells were all capable of producing this material upon superinfection with A-12. Transformed and tumor cell lines however did not appear to elaborate as much early antigen upon superinfection as primary cultures infected with A-12, as judged by the quantity and brilliance of the material stained with sera from tumor bearing hamsters.

In frozen sections of baby hamsters, infected in the newborn period and sacrificed for study at 2, 3, 6 and 7 days, no cells capable of elaborating structural viral antigens were found. Only phagocytized cytoplasmic virus in the reticuloendothelial cells of the liver, lymph nodes and bone marrow was observed. Therefore the selection pressure of tissue culture has not eliminated cells capable of more than the abortive cell-virus relation seen *in vitro*.

Discussion. Adenoviruses of human and other origin have been extensively studied for over 10 years, and an excellent brief review of their general characteristics has been presented by Pereira *et al*(17). In general they have been moderately species specific, and the human group has shown serial propagation in nonhuman cells only with a limited number of types, even though a viral CPE is readily produced in some nonhuman cells. Epithelial cells have been more susceptible than fibroblasts among cells of human origin.

The replication of structural viral elements as detected by immunofluorescence takes place entirely within the nucleus, and the first accurate description of the observable events was presented in a study by Coffin *et al*(18) working with frozen sections and canine hepatitis virus.

Our observations of the viral replication cycle of A-12 in a number of cell types

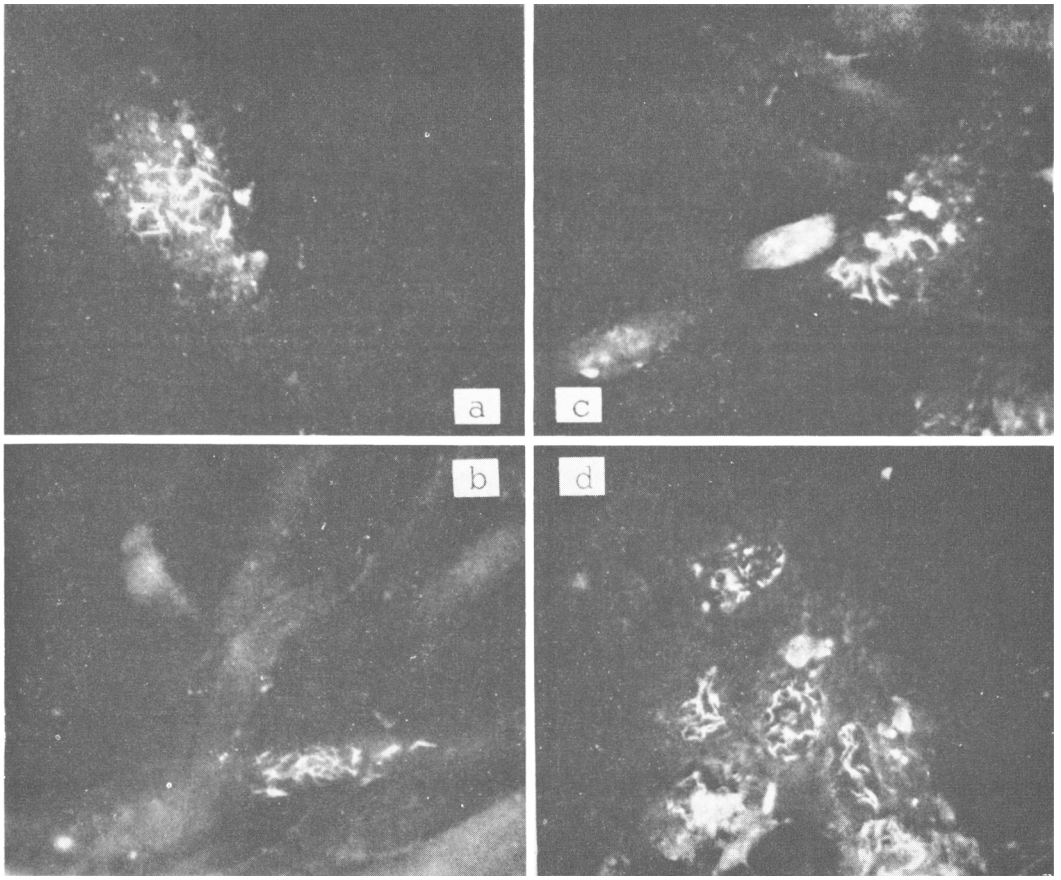

FIG. 4. Demonstration by immunofluorescence of early antigens of adenovirus cycle in non-primate animal cells infected with A-12 and stained at 24 hr. a. baby rabbit kidney; b. baby hamster kidney; c. baby rat kidney; d. baby mouse kidney. Nuclear and cytoplasmic threads and flecks. In c., cells which have rounded up also show diffuse fluorescence (Mag. × 400).

show that in susceptible primate cells, the pattern of accumulation of structural antigenic products resembles that seen in studies of other human adenoviruses(11,12,13). The correspondence of these events to those observed with electron microscopy is still uncertain. The ring-like bodies, opaque or with central diminished density, having a dense peripheral zone and an interior with fine granules and fibrils observed in the electron microscopic studies of Morgan *et al*(19), may be related to the ring forms with satellite dots seen with immunofluorescence. The smooth bright peripheral ring of antigen, stained with antiviral sera, but invisible after staining with acridine orange, may be identical with certain amorphous peripheral material to which ferritin labeled antibody shows affinity in electron microscopic studies. This frequent but by no means universally present peripheral ring of antigen most probably represents structural viral protein, not associated with nucleic acid, accumulating in excess due to asynchronous production of structural viral components. Further work with antisera prepared against individual structural viral components such as those prepared in the studies of Huebner *et al*(8) might resolve this point, since Wilcox *et al*(20) demonstrated that group and type specific viral antigens correspond structurally to capsomeres and fibrils respectively. However, Pope and Rowe(10) saw no differences upon staining infected cells with rabbit sera prepared against purified antigens.

Even among primate cells differences in

production of structural antigens detectable by immunofluorescence were evident, and the brilliance of these products in human fetal cells compared with the diffuse and ill-defined appearance of these same products in S-3 HeLa cells and continuous line amnion is a reflection of the greater efficiency in virus production of human fetal cells. The high proportion of Enders monkey kidney cells capable of elaborating early antigens compared with the small number of cells that go on to produce structural antigens, and the marked adenovirus type CPE that accompanies this situation is good evidence that the cellular alterations resulting in rounding, clumping and detachment from glass are associated with early events in the viral cycle, rather than actual production of virus. This is further reinforced by the observation that non-primate cells, which are capable of elaborating only the early antigens of the cycle develop the adenovirus type CPE and such early antigen production probably requires no viral replication of viral nucleic acid since these antigens are produced in the presence of FUdR(21). As a corollary, it is possible that the events leading to transformation similarly require no viral nucleic acid replication; since transformation occurs in cells which are not observed to produce structural viral antigen.

Particularly interesting are the alterations in cell surface behavior associated with these early events, and the clumping of cells in the adenovirus type CPE may well be related to the marked cohesion of adenovirus transformed cells, and their tendency to grow as balls of cells floating free in the medium when the calcium level is above 0.1 mM as reported by Freeman *et al*(22). The presence of these latter characteristics and the production of early antigens are not interdependent since they were present in our transformed lines of rat cells which do not elaborate early antigens unless superinfected, although they were produced in the initial infection which resulted in transformation. Similarly, the maintenance of the characteristic morphologic and growth properties of transformed cells is not dependent upon continued elaboration of early antigens, since these also were maintained unchanged in our transformed lines of rat cells. At present, the presence of early antigens after infection with A-12 is merely an index of the degree of abortive cell-virus relation achieved, and whether transformation can be established in cells, like the Earle L cell, where the relation does not proceed even to this point remains to be determined.

Summary. By means of immunofluorescence, the sequence of accumulation of antigenic components of the adenovirus cycle was studied in a variety of cells of primate and non-primate origin after infection with adenovirus type 12 (A-12). Structural viral antigens were seen only in primate cells, and most clearly in human fetal kidney, following the appearance of the early antigens. Structural viral antigen was found in a smaller proportion of monkey cells than human cells, even though a high proportion of monkey cells elaborated large amounts of the early antigens. In non-primate cells, rabbit, hamster, rat and mouse cells readily produced early antigens after infection with A-12, but few cells with early antigen were demonstrable in chick cells, and none in Earle L cells. Among A-12 transformed cell lines, and A-12 induced hamster tumor lines in culture, considerable variation in the continuous elaboration of early antigens was found, with a rabbit transformed line and some hamster tumors producing moderate amounts of early antigens, other hamster tumor lines less, and none at all demonstrable in the 3 rat transformed lines studied. It was concluded that the adenovirus type CPE is probably associated with early events in the viral cycle, since it appeared in cells incapable of producing structural viral antigens, and that the viral-induced events leading to transformation may similarly be those occurring early in the viral cycle. Production of early antigens is not a prerequisite for maintenance of the abnormal morphology and growth properties of the A-12 transformed cell, but if present is merely a marker of residual viral information.

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The Incorporation of $S^{35}O_4$ into Bile of Chicks.* (30792)

W. G. MARTIN AND H. PATRICK
Department of Agricultural Biochemistry, West Virginia University, Morgantown

The utilization of inorganic sulfate by the chick to synthesize taurine is of interest since it has been previously reported that cysteine-sulfur does not arise from sulfate(1). Further, over one-half of the sulfate-S^{35} administered to the 24-hour embryo was found in the taurine of the day-old chick(2).

The bile acids, cholic and derivatives of it, conjugate with taurine in the chick liver and are passed into the gall bladder. Cholic acid is the limiting factor in formation of taurocholate in dogs(3), and continued feeding appeared to force the synthesis of taurine(4). Feeding cholic acid to young chicks stimulated the incorporation of sulfate-S^{35} into taurocholate(5).

The purpose of this investigation was to ascertain the influence of taurine and the cholic acid derivatives on incorporation of sulfate-S^{35} into the bile of chicks.

Methods. Day-old chicks were fed *ad libitum* a simplified basal ration of cerelose (glucose-hydrate) and isolated soybean protein with supplements of vitamins, minerals, and corn oil. These birds were maintained on this diet with the taurine and bile acid supplements indicated in the respective Tables for 14 days or as long as indicated. Following the feeding period the isotope was administered and the chicks sacrificed as described in the Tables.

Sulfate-S^{35} was administered orally by means of a 1 ml pipette or injected subcutaneously. Each dose contained 20 μc of carrier free $H_2S^{35}O_4$ in dilute HCl solution. Bile fluid was removed from the gall bladder or the chicks and pooled for each treatment.

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