Morphology and Distribution of SV40-Infected Cells in Human, Simian and Hamster Renal Cell Cultures as Detected by Immunofluorescence.* (28059)

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Simian vacuolating virus, SV40, can be propagated in primary monolayer cultures of renal cells derived from human beings, rhesus and grivet monkeys, and Syrian hamsters(1, 2,3,4,5,6). As the virus multiplies in these systems, viral antigen is produced in the nuclei of infected cells and can be detected by immunofluorescence(5,6). Certain characteristic similarities and differences in the immunofluorescence associated with SV40 replication in these different cell systems are described here.

Material and methods. SV40 virus. Stock virus from the second human fetal kidney passage of SV40 strain VA 45-54 GMK 6/9/61 originally obtained from Dr. M. R. Hilleman and titering $10^{6.5}$ TID 50/0.1 ml in grivet monkey kidney cultures was employed as inoculum for all cultures. *Tissue cultures.* Primary monolayer cultures of grivet monkey kidney, rhesus monkey kidney, newborn Syrian hamster kidney (both primary infected and SV40 transformed), and fetal human kidney (both primary infected and SV40 transformed) were prepared and maintained on coverslips in Leighton tubes as previously described(5,6). Coverslips were removed 10 days after inoculation of SV40, washed in cold phosphate buffer (pH 7.2), fixed in cold acetone for 10 min, dried and stored at –20°C in screwcap tubes until stained. *Antisera.* Rabbits were immunized by the method of Hilleman *et al.*(1) with supernatant fluid from grivet monkey kidney tissue cultures infected with SV40. Serum globulin was precipitated with half saturated ammonium sulfate, and fluorescein labeled with fluorescein isothiocyanate at a ratio of 3% of the antibody protein weight as determined by ultraviolet adsorption at 280/260 mμ. Labeled antibody was eluted from a DEAE column by sodium phosphate solutions of increasing molarity as described by Peters *et al.*(7). Fraction I eluted at 0.5 M sodium phosphate and found to contain the bulk of the antibody was concentrated to original serum volume, tested for specificity and stored in aliquots at –70°. *Immunofluorescence.* Both direct and indirect Coons technics were used. In either case, infected and uninfected cultures were stained with antisera overnight at 4°C, with addition of a small amount of fresh guinea pig complement. *Specificity of immunofluorescence.* Changes described occurred only in infected cultures stained with anti-SV40 sera and were not seen in uninfected cultures. Fluorescein labeled rabbit anti-SV40 sera did not stain cultures infected with other viruses. Dilution or omission of unlabeled anti-SV40 sera used in the indirect Coons technic abolished the specific staining. Substitution of rabbit antibody made against other antigens (bovine serum albumin, diphtheria toxin, polyoma virus) gave no reaction in either the indirect or direct system. Denaturation of SV40 antigen by paraffin at 56°C abolished the specific staining. A rabbit anti-SV40 serum kindly supplied by Dr. M. Hilleman for use in the indirect Coons technic stained the same structures as our rabbit anti-SV40 sera.

Results. Stained coverslips from each tissue fixed on the 10th day after SV40 inoculation were compared with respect to morphology and distribution of cells exhibiting specific immunofluorescence, brilliance of fluorescence, and proportion of cells exhibiting fluorescence. Observations are illustrated in Fig. 1.

Grivet monkey kidney (GMK). In GMK cultures, specific nuclear fluorescence was very brilliant. Infected cells occurred in ran-

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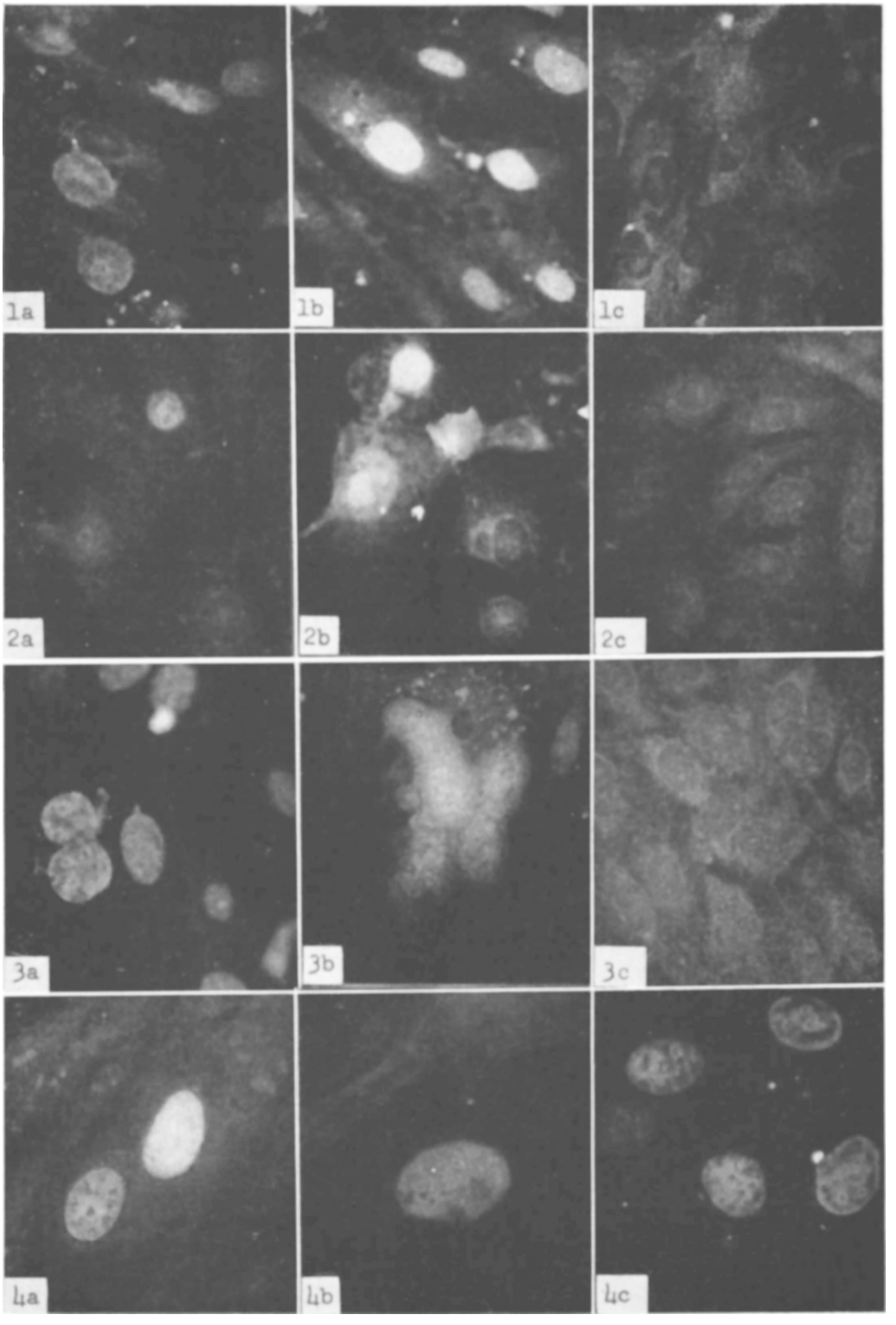
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domly distributed extensive colonies which appeared to arise from separate foci of infection. At 10 days, about 5-10% of cells contained brilliantly stained antigen, distributed first as irregular granular aggregates about pale staining nucleoli with narrow non-staining haloes which are seen in normal cells. Later denser aggregates were concentrated about what still appeared to be nucleoli, though these were now dark and non-staining, giving the impression that the nucleolar substance had been altered. In a still later stage fine granules filled the nucleus, with irregular non-staining areas present in the nucleus in addition to the very dense perinucleolar concentrations. Nuclei exhibiting specific fluorescence were round or oval and often somewhat enlarged. Cytoplasmic vacuoles characteristic of CPE induced by SV40 in GMK cultures appeared as non-staining empty spaces after immunofluorescent staining.

Rhesus monkey kidney (RMK). In infected RMK cultures, no cytopathic changes were recognized in living or hematoxylin-eosin stained cultures. However, when stained by immunofluorescent technic at 10 days after viral inoculation, 5-10% of cells exhibited SV40 specific nuclear fluorescence as brilliant as that observed in GMK cultures. Cells with viral antigen similarly occurred in foci. As in the GMK cultures, antigen first appeared as an irregular faint perinucleolar aggregate, gradually becoming a dense "snowball" around the nucleolus until the concentration of antigen obscured all nuclear detail.

Syrian hamster kidney (SHK). In infected cultures of newborn hamster kidney cells, about 1% of cells exhibited very faint specific nuclear fluorescence when stained 10 days after viral inoculation. Although nuclear fluorescence was much fainter than in GMK and RMK cultures, the same granules, irregular non-staining areas, and concentration about what were regarded as altered nucleoli were easily recognized. Nuclei with such changes were round or oval, often greatly swollen, with a tendency to occur in small groups. In addition, scattered syncytia, containing 3-10 nuclei with the faint granular fluorescence and nuclear morphology comparable to that of single infected nuclei, were seen in infected cultures.

SV40 transformed Syrian hamster kidney cells. As has been reported(6), immunofluorescent staining of such cells leaves one in doubt as to whether any viral antigen is pro-

FIG. 1. SV40 infected, uninfected, and SV40 transformed cultures of grivet monkey, rhesus monkey, hamster and human origin, stained with fluorescent anti-SV40 globulin. Magnification 400×.

1a. SV40 infected grivet monkey culture showing nucleolar staining, non-staining perinucleolar halo and early perinucleolar SV40 antigen concentration.

1b. Same, massive nuclear antigen accumulation, perinucleolar concentration. Cytoplasmic non-staining vacuoles.

1c. Same, uninfected. Overexposed to show perinucleolar non-staining halo, slight nonspecific nucleolar, nuclear and cytoplasmic staining.

2a. SV40 infected rhesus monkey kidney culture, lower cell apparently uninfected, middle cell shows perinucleolar ring of antigen, upper cell shows antigen accumulation around altered non-staining nucleolus.

2b. Same, dense accumulations around central nucleoli in "snowball" effect. Two lower cells with nucleoli stained, non-staining perinucleolar halo, and faint early perinucleolar antigen concentration.

2c. Same, uninfected. Non-staining perinucleolar halo, slight nonspecific nucleolar and nuclear staining.

3a. SV40 infected hamster newborn kidney culture. Heavily overexposed photograph to show faint SV40 antigen accumulation around altered non-staining nucleoli, round or oval shaped nuclei associated with SV40 infection.

3b. Same, syncytium formation. Faintly staining nucleoli, non-staining perinucleolar halo and faint granular nuclear antigen.

3c. Same, SV40 transformed culture. Overexposed to show faint nuclear staining.

4a. SV40 infected primary human fetal kidney culture. Apparently uninfected cells with faint staining nucleoli. One infected cell shows antigen concentration around altered non-staining nucleolus, and irregular nuclear non-staining areas. The other shows dense nuclear antigen.

4b. Same, swollen oval degenerating cell with fainter staining antigen.

4c. Same, SV40 transformed superinfected culture. Perinucleolar antigen, non-staining altered nucleoli, variable intensity of staining, round or oval nuclei.

duced. Faintly staining nucleoli with non-staining narrow haloes and faint amorphous staining of nuclear substance were seen. After superinfection, no nuclei with the characteristic perinucleolar immunofluorescent pattern noted in primary cultures were observed. Scattered syncytia containing 3-10 nuclei similar to those observed in primary infected cultures but with fainter, more diffuse fluorescence were seen in both "uninfected" and superinfected cultures.

Human fetal kidney (HFK). In infected human fetal kidney cultures, specific nuclear fluorescence was less brilliant than in grivet or rhesus monkey cultures, but far more brilliant than in hamster cells. In cultures fixed 10 days after viral inoculation, about 1% of cells exhibited specific fluorescence. Distribution of granular fluorescence in the nuclei of these cells resembled that seen in GMK cultures, but a higher proportion of such cells were oval and some were greatly swollen. Swollen cells with specific fluorescence were less brilliant than those of more normal size. Cells with antigen occurred usually in small aggregates, randomly distributed.

SV40 transformed human fetal kidney cells. As previously reported(5) about 0.01 to 0.1% of nuclei in such cultures showed bright specific fluorescence 10 days after SV40 superinfection. These cells exhibited the characteristic perinucleolar aggregations of antigen observed in primary HFK cultures and were often difficult to detect, since most of the transformed cell nuclei presented the characteristic oval shape associated with SV40 antigen production in primary cultures, and a diffuse faint fluorescence not seen in uninfected cultures.

Discussion. Nuclear localization. Localization of specific SV40 fluorescence in the nuclei of infected cells in all 4 tissues supporting SV40 replication suggests that in each, virus multiplies within the nucleus. Furthermore, aggregation of antigen around what appeared to be altered nucleoli provides additional evidence that the nucleolus is involved in SV40 replication. Previous electron microscopic studies(2,8) have indicated that SV40 particles appear in the region of the nucleolus and that the nucleolus is altered. Accordingly it may be postulated that the perinucleolar concentration of antigen noted by immunofluorescent staining represents the stream of viral particles expelled from the site of synthesis and assembly within the nucleolus.‡ The irregular non-staining nuclear areas probably represent empty areas seen by electron microscopy in nuclei containing many viral particles.

Grouping of fluorescing cells in colonies. In all cultures cells with specific nuclear fluorescence occurred in groups or clusters randomly distributed throughout the monolayer. In cultures where large clusters are formed (GMK and RMK), fluorescing cells are often contiguous, but in cultures where fluorescing cells occur in small groups (HFK and SHK) individual fluorescing cells may be separated on each side by 2 or 3 non-staining cells. In the latter, therefore, spread of viral infection by way of intercellular bridges, or as multiplication of a single infected cell appears unlikely. Rather the pattern suggests that local increases in viral concentration following release from an infected cell offer a greater opportunity in that area for new infection of susceptible cells. Furthermore, cells appear to vary in capacity to support the type of viral replication which can be detected by immunofluorescent technic. Thus it is possible that other cells, not visibly stained by this technic, may be producing viral antigen in lesser concentrations. Variation in capacity to produce stainable antigen is accentuated in SV40 transformed cells where nuclei display the morphologic alterations characteristic of SV40 infected cells, but appear only rarely to present specific fluorescence.

Brilliance of fluorescence and proportion of cells exhibiting fluorescence. Infected

‡ After completion of this work, we were privileged to see electron microscopic photographs from a detailed study of the role of the nucleolus and the nucleolar chromatin in the replication of SV40 virus by Dr. Nicole Granboulan at the Centre National de Recherches sur le Cancer in Ville-Juif, France, which independently confirm and amplify the conclusions drawn from immunofluoroescent staining.

cells in GMK and RMK cultures exhibited the most brilliant fluorescence, those in HFK and SV40 transformed HFK less, and those in SHK and SV40 transformed SHK least. The proportion of fluorescing cells diminished in the same order. It seems likely that the number of cells with fluorescence approximates the per cent of cells actively producing virus, and that the brilliance of fluorescence is proportional to quantity of virus produced. Evidence supporting these correlations is afforded by results previously reported of a comparative titration of SV40 production in GMK and HFK cultures(5). The end point as determined by back titration in GMK cultures was consistently 2 logs lower in HFK cultures. It is curious that *in vitro* transformation has been observed so far only in the 2 cell systems, HFK and SHK, where the virus appears to propagate less easily. A similar inverse relation between ease of propagation and ease of transformation has been generally observed with polyoma virus in mouse embryo and hamster cultures. Indeed as noted by Fraser and Gharpure in the hamster cell line of Stoker, using polyoma virus at infection multiplicities of 10^8 pfu per cell, a ratio producing a transformation rate of 1-4%, only 0.5% of cells show nuclear multiplication of virus by immunofluorescence although, at 24 hours or less, all cells show cytoplasmic uptake of the agent(9).

Conclusions. In SV40 infected grivet monkey, rhesus monkey, primary fetal human, SV40 transformed fetal human and primary newborn Syrian hamster kidney tissue cultures viral antigen is found in the nucleus. Because of aggregation of antigen around what appears to be altered nucleoli in all 4 tissues it is suggested that viral synthesis and assembly may occur in the nucleolus. In the 4 tissues studied there is an inverse correlation between proportion of cells exhibiting specific nuclear fluorescence, brilliance of fluorescence and ease of induction of transformation *in vitro*.

No specific nuclear fluorescence was observed in SV40 transformed Syrian hamster renal cultures even when superinfected with SV40 virus.

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Nucleic Acid Content of Mammary Glands of Lactating Rats.*† (28060)

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In the rat, histological observations(1) suggested that mammary hyperplasia did not occur after mid-pregnancy, whereas deoxyribonucleic acid (DNA) content, which estimates cell numbers(2), increased throughout pregnancy(3,4,5). Histological studies using colchicine technic(6) revealed little mitotic activity during lactation but later work showed that DNA content(3,4) approxi-

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