*INFECTION OF MUSCLE CULTURES FROM VARIOUS SPECIES WITH ONCOGENIC DNA VIRUSES (SV40 AND POLYOMA)**

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In primarily infected tissue culture cells, oncogenic viruses stimulate the synthesis of cellular DNA[1-6] and of a new antigen unrelated to structural antigens of the virion, referred to as T antigen[7] or induced complement-fixing antigen (ICFA)[8] [a term originally introduced for reasons indicated elsewhere[8a]].[9, 10] The biological significance of the T antigen is still unclear, but its synthesis appears to be relevant, if not necessary, to the malignant transformation of cells.[11, 12] The ability of oncogenic viruses to change the normal function of cellular genomes has previously been tested under experimental conditions in which the mitotic activity and synthesis of cellular DNA were partially repressed. This repression was either temporary, as caused by contact inhibition, or it was the result of permanent damage of the cell by X ray.[1, 5] It has also been shown that the competence of the cell to synthesize viral and cellular DNA and T antigen is dependent on the physiologic condition of the cell at the time of infection.[13, 14]

The problem considered in the present work is whether viral and viral-related substances may be induced by oncogenic DNA viruses in cells in their terminal state of differentiation (G_0)[15] which have lost the capacity to synthesize DNA and to divide. For this purpose we have chosen muscle fibers (myotubes) which form *in vitro* or *in vivo* by fusion of mononucleated cells (myoblasts). The myotubes are characterized by a large number of nuclei in which DNA synthesis and mitotic activity are repressed.[16-21] Hamster, rat, mouse, and human muscle cultures were infected with either polyoma (PV) or SV40 viruses and examined with regard to the following questions: (1) Can virus functions (induction of cellular DNA synthesis, T-antigen and virus synthesis) be expressed in the multinucleated myotube as they can in the single-nucleated cells? (2) Do the nuclei in a myotube respond to the infection as discrete units or as a complex?

Materials and Methods.—Viruses: Wild[10] and small plaque[22] strains of polyoma were routinely propagated in mouse embryo fibroblasts; both pools had titers of $10^{8.5}$ PFU/ml. SV40 (strain RH911), with a titer of 10^9 PFU/ml, was kindly supplied by F. Jensen (The Wistar Institute).

Tissue cultures: Muscle cultures were prepared from randomly bred hamster, inbred rat (strain DA/Ss), and mouse (strain C_3H) embryos, and from human fetuses according to methods previously described.[17-21] Skeletal muscle tissue was excised from hamster, rat, and mouse embryo limbs taken about 2 days before delivery, and from the extremities of 7- to 10-week-old human fetuses. Tissue fragments about 1–2 mm in diameter were trypsinized for 30 min at 37°C in a 1% trypsin solution (calcium- and magnesium-free); the cell suspension was then seeded at a concentration of 2–3 $\times$ 10^6 cells in 5 ml of medium per plastic Petri dish (60 $\times$ 16 mm) containing cover slips. The culture medium was Earle's balanced salt solution with twice the concentration of amino acids and vitamins in Eagle's basal medium and supplemented with 10% horse serum (Microbiological Associates, Bethesda, Maryland). The hamster, rat, and mouse cultures were grown in 10% CO_2 atmosphere at 37°C throughout the experiment. The human muscle cultures were grown in 10% CO_2 atmosphere for 2–3 days and, 1 day before infection, were transferred to an incubator with a flow of 5% CO_2 in air. Unless otherwise stated, cultures were infected at various times after plating at a multiplicity of 50 PFU/cell.

Immunofluorescence: The methods for staining for T antigen and viral protein (VP) antigen

have been previously described.[8, 10] Antipolyoma T serum was kindly supplied by Dr. R. Gilden (Flow Laboratories, Rockville, Maryland). Baboon anti-SV40 viral and hamster anti-T sera labeled with fluorescein-isothyocyanate were obtained from Flow Laboratories, Rockville, Maryland. The cover slip cultures were fixed at −20°C in an acetone-methanol mixture (7:3).[10] Twenty-five % glycerol in 0.01 *M* phosphate buffer-saline solution, pH 7.6, was used as mounting medium.[23] Nonspecific staining was excluded by appropriate controls.[10] The proportion of positive cells in the PV-infected cultures was determined by counting 1000 cells in two samples. In the SV40-infected cultures, because of the low number of infected cells, 50 microscope fields in two cover slips were scored, each field containing an average of 100 cells.

Radioautography: Cultures were exposed to H^3-TdR (6 c/mmole) at a concentration of 2 μc/ml for 8 hr prior to fixation. The cover slips were fixed in Carnoy's solution for 30 min and kept overnight in 70% ethanol. The slides were coated with Kodak NTB3 emulsion and the radioautographs developed after 8–10 days. The slides were then stained either with methyl green or hematoxylin-eosin.

Results.—Shortly after plating, cultures consisted of mononucleated, bipolar fibroblast-like cells. As the cultures became confluent, long ribbon-like cells containing multiple nuclei (myotubes) appeared.[24] The time of appearance of the myotubes varied according to the density of the culture, the conditions of incubation, and the species of origin, but was usually three to five days after plating. The myotubes then progressively increased in length and ramification.

When control cultures were exposed to H^3-TdR within 24–48 hours after plating (before myotube formation), a certain proportion of cells showed incorporation of the labeled precursor. When the cultures were exposed to H^3-TdR after formation of the myotubes, no multinucleated cells were labeled. Similar results were obtained with cultures from all four mammalian species, which confirms the observation that DNA synthesis does not occur in the myotubes.[18, 19, 21]

Polyoma-infected hamster and rat muscle cultures: (a) *Induction of PV-T antigen and PV antigen:* When the muscle cultures were infected at the stage of single nucleated myoblasts with a multiplicity of 50 PFU/ml, about 40 per cent of the hamster and 25 per cent of the rat cells were positive for the T antigen within three days postinfection (Table 1). This proportion of positive cells is similar to that observed for whole rat and hamster embryo cell cultures infected at the same multiplicity.[25] Only very few nuclei stained for the viral antigen, confirming the observation that a large majority of hamster and rat cells undergo an abortive infection with polyoma virus with little formation of virion.[10] The muscle culture, in which a large proportion of cells are myoblasts, therefore showed the same sensitivity to PV infection as fibroblast cultures from the same species.

When the cultures were infected after the majority of myotubes had already formed, T antigen occurred only in the single-nucleated cells. Only rarely was a small myotube positive for the antigen. The density of myotubes in the rat cultures was frequently so high as to cover virtually the whole surface of the cover slips.

TABLE 1

PERCENTAGE OF T AND VIRAL ANTIGEN POSITIVE MUSCLE CELLS

	Polyoma			SV40
Antigens	Hamster	Rat	Mouse	human
T	39.5	25	61	0.6
Viral	0.9	0.5	22	0.25

Hamster, rat, and mouse mononucleated skeletal muscle cells were infected with polyoma virus at a multiplicity of 50, human muscle with SV40 at a multiplicity of 40. Hamster and rat culture were stained for specific immunofluorescence on the third day, mouse culture on the second day, and human culture on the fourth day postinfection.

In such cases only sparse, single cells showed specific immunofluorescence. It is apparent that hamster and rat muscle cells after fusion and formation of myotubes are no longer sensitive to PV infection.

The presence of rare, antigen-containing myotubes in some cultures could have resulted from induction of the T antigen in myoblasts just before they were incorporated into the myotubes. Direct evidence for this was obtained in experiments in which the cultures were infected just a few hours before cell fusion began and were assayed at various intervals thereafter. In such a case, myotubes with large numbers of antigen-containing nuclei were present. The antigen usually appeared in groups of adjacent nuclei in one section but, occasionally, all nuclei throughout the whole length of the myotube were immunofluorescent. Similar results were obtained in rat and hamster myotubes (Fig. 1). About 50 per cent of hamster myotubes were positive at 32 hours, and nearly 80 per cent at 44 hours postinfection. In the rat cultures 50 per cent of myotubes contained the antigen at 44 hours postinfection.

No viral antigen was detected in either the hamster or rat myotubes infected with the wild type of polyoma virus, while occasional myotubes had viral antigen when infected by the small plaque strain.

(b) *Induction of DNA synthesis:* Results similar to those described for the induction of T antigen were obtained in studies of the induction of DNA synthesis in the PV-infected hamster and rat muscle cells. No DNA synthesis took place if the cultures were infected after the formation of the myotubes. When cultures were infected prior to fusion, virtually 100 per cent of hamster myotubes at 32 hours after infection (Fig. 2) and about 50 per cent of rat myotubes at 44 hours after infection had labeled nuclei. As in the case of the T antigen, all nuclei in a single myotube or groups of nuclei in a section of the cell were labeled. However, the intensity of labeling was weaker than in the single-nucleated cells. Induction of DNA synthesis in rat muscle cultures infected by PV has been recently reported.[26]

Polyoma-infected mouse muscle cultures: About 60 per cent of single-nucleated mouse muscle cells infected with PV were positive for the T antigen at 48 hours after infection. In contrast to rat and hamster cells, mouse cells readily supported PV replication and 22 per cent of mouse cells stained positively for the viral antigen (Table 1). Infection of myoblasts prior to fusion resulted in the appearance of myotubes positive for T antigen (20–40%) (Fig. 3). A small proportion of mouse myotubes was also positive for viral antigen (Fig. 4). Fully formed myotubes were not susceptible to infection.

The frequency of myotubes with H^3-TdR-labeled nuclei was much lower ($\leqslant 0.5\%$) in PV-infected mouse muscle cultures than in the rat and hamster cultures. Two patterns of labeling were observed: in some of the myotubes the labeling was as intense as in the mononucleated cells (Fig. 5), while in others it was as light as in the hamster and rat cells. The low percentage of H^3-TdR-labeled myotubes may be due to the fact that PV is lytic for mouse cells; many of the myoblasts were probably damaged or destroyed prior to fusion. If these cultures were maintained for six to seven days, then complete lysis occurred, including the myotubes.

SV40-infected human fetus muscle cultures: In cultures of human fibroblasts, even when infected by SV40 at high multiplicity, only a small proportion of the population (about 1%) shows evidence of infection, as estimated by the proportion

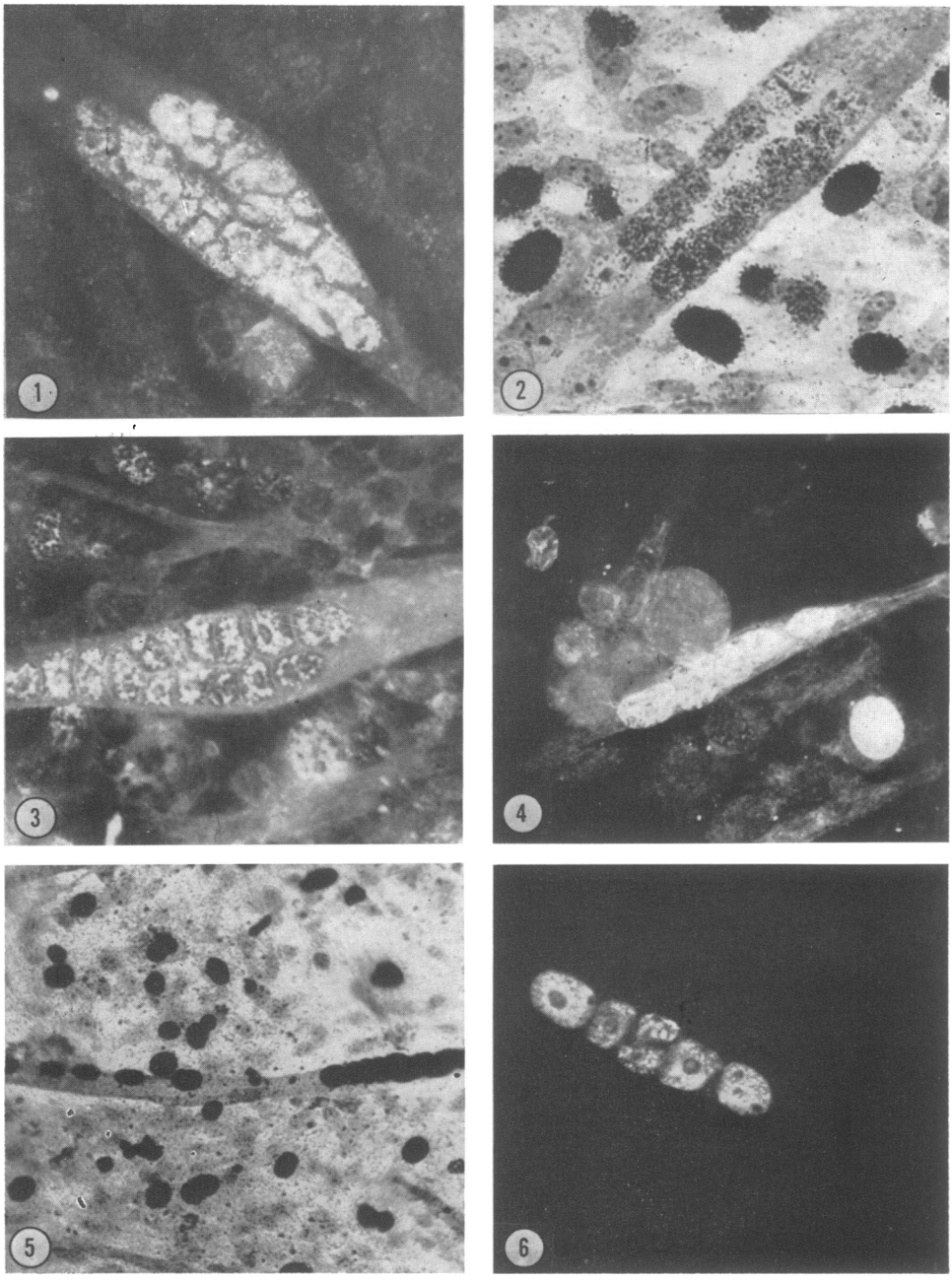

FIG. 1.—Hamster myotube stained for polyoma T antigen 52 hr postinfection. × 400.
FIG. 2.—Hamster myotubes. Incorporation of H^3-TdR at 32 hr after polyoma virus infection. × 400.
FIG. 3. Mouse myotubes stained for PV-T antigen 28 hr postinfection. × 400.
FIG. 4.—Mouse myotubes stained for PV-viral antigen 28 hr postinfection. × 400.
FIG. 5.—Mouse myotubes. Incorporation of H^3-TdR at 48 hr after polyoma virus infection. × 240.
FIG. 6.—Human myotube stained for SV40 T antigen 4 days postinfection. All the nuclei which are positive in this photograph belong to a single myotube; the background cytoplasmic staining was too faint to be reproduced. × 400.

of cells that stain for the SV40-T and viral antigens.[13] As shown in Table 1, 0.6 per cent human muscle cells infected at a multiplicity of 40 were positive for T antigen and 0.25 per cent for viral antigen four days after infection. Both antigens appeared in the human muscle cells relatively late, not earlier than two days, after infection. Despite the low proportion of single cells infected, some human myotubes showed multinuclear immunofluorescence for the T antigen (Fig. 6). Very few myotubes positive for viral antigen were found on the numerous cover slips examined. Few human myotubes (0.1–0.2%) showed thymidine uptake when infected prior to fusion.

Discussion.—The results presented indicate that skeletal muscle cells are as susceptible and responsive to infection by polyoma and SV40 viruses as other mononucleated cells of the same species. Moreover, while these cells are synthesizing cellular and viral DNA, T antigen, and, in some instances, viral antigen, they maintain the competency to take part in the formation of highly differentiated multinucleated myotubes. It has been shown that chicken myoblasts infected with Rous sarcoma, an RNA virus, remain capable of fusing and forming multinucleated myotubes.[27, 28] DNA synthesis is repressed in myoblasts prior to fusion; indeed, arrest in G_0 appears to be the prerequisite for the process of fusion and differentiation.[21] Yet it is quite obvious that both polyoma and SV40 may induce DNA synthesis in these cells without interference with their fusion. Since infection of hamster and rat cells with polyoma virus is rarely of the productive type,[10] it may be assumed that cellular DNA synthesis is induced in these cells. Thus polyoma and, most probably, SV40 are able to rescue these particular cells from G_0 into S phase.

Failure to demonstrate infection of fully formed myotubes could be due to either of two reasons: (*a*) the nuclei of the myotubes are irreversibly repressed; or (*b*) the myotubes have an altered cell surface. The first hypothesis appears to be rather improbable, since the T antigen may be induced even when DNA synthesis is inhibited.[8, 12, 29] The second hypothesis is more probable since the process of differentiation in muscle cells is accompanied by formation of contractile proteins[21] and by the disappearance of a surface antigen present in the myoblasts prior to fusion.[30] It has been shown that mouse metanephrogenic mesenchyme during differentiation into tubules becomes resistant to polyoma virus infection.[31] To clarify whether the resistance of myotubes to PV and SV40 infection is due to surface changes, it would be interesting to investigate whether these cells are sensitive to viral infectious nucleic acids.

One of the most interesting problems that emerges from the present studies is the mechanism by which the infecting virus induces synchronous changes in entire groups of nuclei within one myotube even when the nuclei are far apart. It could be assumed that, at the high multiplicity of virus used, all the myoblasts that are incorporated in the myotubes were infected, but this seems improbable since not all the mononucleated cells were infected. Furthermore, in some sections of a single myotube, all the nuclei were induced for synthesis of DNA and T antigen, while in other sections entire groups remained negative. In the case of SV40 and human myoblasts, no more than 1 per cent of the mononucleated cells with random distribution in the culture stained for T antigen. The probability is very low for only infected cells of such cultures to meet and fuse. It seems more reason-

able to conclude that the information for the synthesis of virus-specific products was brought into the myotube by one infected cell which transferred it to the rest of the nuclei by chain induction or derepression. Alternatively, the cells initially "switched on" may serve as a donor of specific products that are distributed to and taken up by other nuclei.

Transfer of the T antigen from SV40-transformed virus-free human cells into nuclei of rat myotubes, into which the human cells were incorporated, has been recently shown.[32]

Summary.—Hamster, rat, and mouse muscle cultures were infected with polyoma, and human muscle cultures with SV40 viruses; they were then tested for induction of T antigen, viral antigen, and for DNA synthesis. Mononucleated myoblasts were as susceptible to infection as whole embryo fibroblasts of the same species. Myoblasts from all the species infected prior to fusion were capable of forming multinucleated cells (myotubes) in which either large groups of adjacent nuclei or all the nuclei contained T antigen. A great proportion (50–100%) of hamster and rat myotubes containing at least one myoblast infected prior to fusion showed DNA synthesis, presumably cellular, while DNA synthesis was completely repressed in the noninfected myotubes. Viral antigen was rarely found in myotubes of all four species. Fully formed myotubes were resistant to infection.

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