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Modification of Cultured Rabbit Cells by Ultraviolet-Inactivated Noncytocidal Shope Fibroma Virus (39843)[1]

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Upon infection with noncytocidal strains of Shope fibroma virus (SFV), an oncogenic poxvirus (1), serially cultured domestic rabbit kidney (DRK) cells survive, continue to proliferate, and acquire altered characteristics (2, 3). The infected cells enlarge, develop a fibroblastoid morphology, and lose their normally strict contact-inhibition of movement to form multilayered cellular aggregates (2, 4). In addition, the cells convert to a state in which replication of vesicular stomatitis virus (VSV) and several other unrelated RNA viruses is dramatically facilitated (5). Hence, interaction between SFV and DRK cells *in vitro* provides a useful experimental model to investigate mechanisms by which viruses induce cellular modifications. The various modifications induced by SFV in DRK cells were previously examined under conditions that permitted complete SFV replication. To define more clearly the viral replicative events required to cause these cellular alterations, therefore, it was of interest to determine whether the virus can induce similar changes during abortive infection. In the present report, the ability of SFV inactivated by ultraviolet (uv) light to alter the cultural behavior to DRK cells and to facilitate the replication of VSV was investigated.

Materials and methods. Cloned DRK_3 cells originally isolated from the kidneys of a New Zealand white rabbit (2) were grown in medium 199 supplemented with 10% heat-inactivated calf serum, 0.25% lactalbumin hydrolysate, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin. Media for sealed cultures and unsealed cultures incubated in 5% CO_2 were buffered with sodium bicarbonate at respective concentrations of 0.075 and 0.23%. Vero cells derived from African green monkey kidney tissue were supplied by Y. Wong, State Hygienic Laboratory, Iowa City, Iowa. These cells were grown in Eagle's minimal essential medium (MEM) containing 5% heat-inactivated fetal bovine serum, 0.23% sodium bicarbonate, and the usual amounts of penicillin and streptomycin.

The Patuxent strain of SFV from the American Type Culture Collection was grown in monolayers of DRK_3 cells infected at a multiplicity of 1-5 infectious particles/cell. Pooled stocks assayed in DRK_3 cultures as previously described (2) contained $2\text{-}5 \times 10^7$ focus-forming units (FFU)/ml. The Indiana strain of VSV was obtained from Dr. J. E. Rodriguez, University of Iowa. Suspensions containing $1\text{-}4 \times 10^9$ plaque-forming units (PFU)/ml when assayed in Vero cultures were prepared by infecting monolayers of Vero cells at a multiplicity of 10^{-5} PFU/cell. This low multiplicity was used to prevent the production of defective VSV particles (6).

To examine effects of uv-irradiated SFV on cellular behavior in culture, DRK_3 cells were grown in shell vials containing glass coverslips as described previously (2). Each vial was seeded with $1\text{-}2 \times 10^5$ cells in 2 ml of growth medium 199. After incubation sealed at 37° for 2-3 days, the cultures were renourished with maintenance medium 199 containing calf serum reduced to 2.5% and sodium bicarbonate increased to 0.23%. The cultures were then routinely incubated for an additional 1-2 days at 35° to establish uniform monolayers of $4\text{-}5 \times 10^5$ contact-inhibited cells. At this time the cultures were inoculated with 0.5 ml of various viral suspensions subjected to increased dosages

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of uv radiation. Controls included cultures mock-infected with medium alone or inoculated with nonirradiated virus diluted in medium to mimic the decreased multiplicity of infection associated with uv inactivation of the virus. Following viral adsorption at 37° for 3 hr, 2 ml of maintenance medium 199 was added to each culture, and incubation was continued at 37°. At intervals, representative cultures were stained with hemotoxylin and eosin to observe cellular morphology, with fluorescein-labeled anti-SFV serum to detect viral antigens, or with acridine orange to visualize cytoplasmic inclusions of SFV DNA. These latter two methods were used to permit quantitation of DNA and antigen-positive cells.

To investigate facilitation of VSV, DRK_3 cells were grown in glass screw-capped tubes (15 × 125 mm). Each tube was seeded with 1×10^5 cells in 1 ml of growth medium 199. After incubation at 37° unsealed in 5% CO_2 for 2 days, the cultures routinely contained $2\text{–}3 \times 10^5$ actively growing cells. These were inoculated with 0.2 ml of virus suspended in Tris-buffered saline (TBS, pH 7.4). Cultures receiving both SFV and VSV were inoculated simultaneously with a mixture of the two viruses. After viral adsorption at 37° for 2 hr, the cultures were washed twice with 5 ml of TBS, renourished with 1 ml of maintenance medium 199, and again incubated at 37°. At this time and at 24-hr intervals thereafter, sample cultures were placed at −55° to be assayed later for infectious VSV.

Infectious VSV was quantitated by inoculation of confluent Vero cell monolayers. Infected DRK_3 cultures to be assayed for VSV were thawed and treated for 30 sec in a Branson sonifier (Cole-Parmer Instruments & Equipment Company). The culture fluids were then serially diluted in cold TBS. Vero monolayers were inoculated with 0.1 ml of these various dilutions and were incubated at 37° for 2 hr to allow viral adsorption. Each culture was then overlayed with MEM containing 5% heat-inactivated fetal bovine serum, 0.5% methylcellulose (Matheson, Coleman, and Bell), 0.23% sodium bicarbonate, and the usual antibiotics. Within 24 hr at 37°, VSV plaques were readily countable with the aid of a dissecting microscope. The presence of SFV in the dual-infected cultures had no affect on VSV plaque formation.

Suspensions of SFV to be uv irradiated were diluted in growth medium 199 and were placed in glass petri dishes (90 mm). Volumes of 5 ml were exposed with constant agitation to uv light at an intensity of 3.17 $\mu W/cm^2$. Exposure for 25 sec routinely reduced the titer of infectious SFV by a factor of 10^{-3}. In viral suspensions irradiated for 100 sec, no surviving infectious SFV was detectable.

Results. The effect of uv-irradiated SFV on the cultural behavior of DRK_3 cells is shown in Fig. 1. Nonirradiated virus at an input multiplicity of 5 SFV FFU/cell caused typical (2) changes in cellular behavior and appearance (Fig. 1a). Multilayered aggregates of elongated cells were present throughout the cultures. Similar though somewhat less dramatic effects were seen in cultures inoculated with the same suspension of SFV after it was uv irradiated for 25 sec to reduce the multiplicity of infection from 5 to 0.001 FFU/cell (Fig. 1b). In parallel cultures inoculated with nonirradiated virus diluted to give a comparable multiplicity of 0.005 FFU/cell (Fig. 1c), only widely scattered cellular aggregates were present. When the viral suspension was irradiated for 100 sec, infectious SFV was no longer detectable in the inoculum, and the cells appeared unaffected (Fig. 1d). These cultures treated with excessively irradiated virus resembled mock-infected controls (not shown).

The extent of viral DNA and antigen synthesis in these various cultures is presented in Table I. Essentially all of the cells in cultures inoculated with the undiluted, nonirradiated viral suspension contained intracytoplasmic inclusions stained yellow-green by acridine orange. Such inclusions represent synthesized poxviral DNA (7). Similar numbers of cells in these cultures were synthesizing major SFV-specific antigens detected by fluorescein-conjugated rabbit γ-globulin prepared from rabbits hyperimmunized with infectious SFV. In the cultures inoculated with SFV uv irradiated for 25 sec, despite the widespread effect on cellular behavior seen in Fig. 1b, clusters of cells

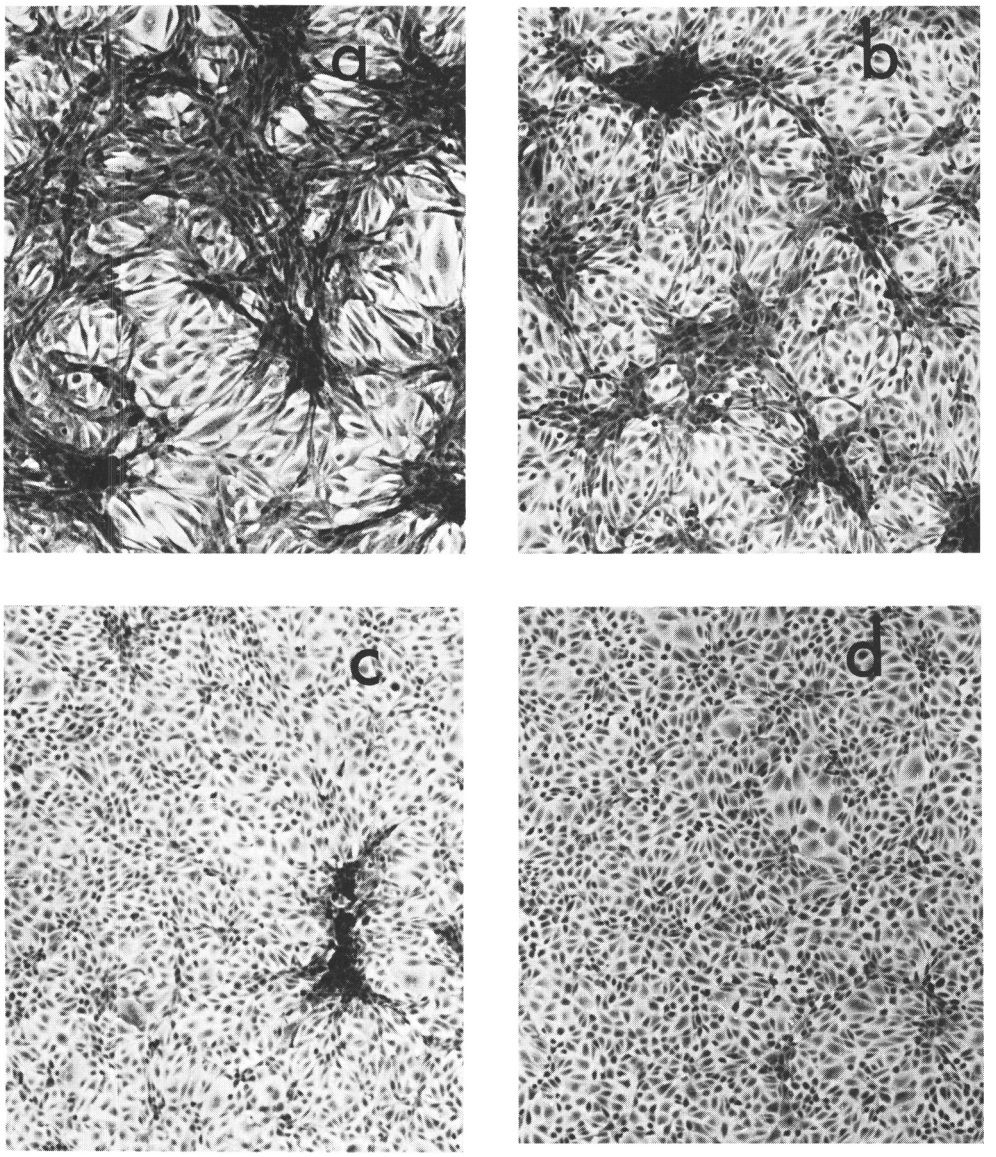

FIG. 1. Cultural characteristics of DRK_3 cells after treatment with various preparations of SFV. Confluent monolayers of cells were inoculated and then examined after 3 days at 37°. (a) Cells inoculated with nonirradiated SFV at a multiplicity of 5 FFU/cell. (b) Culture treated with the same SFV suspension as in (a), but uv irradiated for 25 sec to reduce its infectivity to 0.001 FFU/cell. (c) Cells treated with the nonirradiated suspension of SFV virus diluted to contain 0.005 FFU/cell. (d) Monolayer of cells inoculated with SFV uv irradiated for 100 sec to abolish infectivity. Hematoxylin and eosin stain. ×50.

in only a few of the many cellular aggregates contained detectable inclusions of viral DNA or specific viral antigens. Similar numbers of these DNA and antigen-positive foci were present in the cultures inoculated with nonirradiated virus diluted to provide a comparable input multiplicity of infection. No cells synthesizing detectable viral components were found in the mock-infected controls or in the cultures treated with virus irradiated for 100 sec.

In addition to alteration of cellular morphology and behavior, uv-irradiated SFV also facilitated the replication of VSV in

cultures of actively growing DRK_3 cells. This is demonstrated by the results in Table II. After simultaneous inoculation with a mixture of nonirradiated SFV plus VSV, more than 4000 VSV PFU/cell was produced in 48 hr. When the same suspension of SFV was uv irradiated at a dosage that reduced the multiplicity of SFV to 0.02 FFU/cell, a comparable yield of more than 3000 VSV PFU/cell was present 48 hr after dual infection. Contrastingly, in cultures inoculated simultaneously with VSV and nonirradiated SFV diluted to give a similar input multiplicity of 0.04 FFU/cell, little if any infectious VSV was produced. Prolonged uv irradiation abolished the ability of SFV to facilitate VSV; in such cultures less than 1 VSV PFU/cell was present after 48 hr. This was comparable to the amount present in control cultures inoculated with VSV alone. Since the amount of residual infectious VSV present at 2 hr after inoculation was between 1 and 10 PFU/cell (not shown), it appeared that no VSV replication occurred in the DRK_3 cultures in the absence of SFV.

TABLE I. SYNTHESIS OF VIRAL DNA AND ANTIGENS IN DRK_3 CELLS TREATED WITH UV-IRRADIATED SFV.

Inoculum[a]	Input SFV (FFU/cell)[b]	Percentage of cells containing: Cytoplasmic DNA[c]	Percentage of cells containing: SFV-Specific antigens[d]
SFV	5	>90	>90
SFV (uv)[e]	0.001	foci[f]	foci
SFV (diluted)	0.005	foci	foci
SFV (uv)[g]	<0.000001	0	0

[a] Same suspension of SFV treated or untreated.
[b] FFU, focus-forming units.
[c] Yellow-green inclusion stained by acridine orange 48 hr after inoculation.
[d] Stained with fluorescein-conjugated rabbit anti-SFV globulin 48 hr after inoculation.
[e] Ultraviolet irradiation for 25 sec.
[f] Widely scattered aggregates containing multiple positive cells.
[g] Ultraviolet irradiation for 100 sec.

Discussion. The results of this investigation extend the previous work of Hinze and Walker (2) and demonstrate that nonreplicating SFV can modify the cultural behavior of DRK_3 cells. In cultures treated with suspensions of SFV partially inactivated by uv light, cells became altered and formed multilayered cellular aggregates. Since the cells in a large majority of these aggregates contained no detectable cytoplasmic viral DNA or viral antigens, either they were not infected with SFV or the infection was abortive. The cellular aggregates that were positive for viral DNA and antigens in these cultures presumably were initiated by infectious SFV present in the partially inactivated viral suspensions. In control cultures treated with nonirradiated SFV diluted to provide essentially the same low multiplicity of infection as in the partially inactivated inocula, all of the few cellular aggregates that formed were comprised of cells synthesizing readily detectable viral components. It is unlikely, therefore, that the low levels of surviving infectious virus present in the suspensions of SFV partially inactivated by uv radiation caused the numerous aggregations of cells found to be negative for viral DNA and antigens. Rather, it appears that cells abortively infected with uv-irradiated SFV became modified and acquired a penchant for aggregation. Based on the generally accepted assumption that nucleic acid is

TABLE II. FACILITATION OF VSV REPLICATION IN DRK_3 CULTURES BY UV-IRRADIATED SFV.

Inoculum[a]	Treatment of SFV[b]	Input of infectious particles/cell: SFV	Input of infectious particles/cell: VSV	Yield of VSV[c] (PFU/cell)
SFV + VSV	None	10	20	4700
SFV + VSV	uv	0.02	20	3600
SFV + VSV	Dilution	0.04	20	1.3
SFV + VSV	uv	<0.000001	20	0.7
VSV			20	0.6

[a] Mixture of SFV and VSV or VSV alone.
[b] Same suspension of SFV nontreated, uv-irradiated at different dosages, or diluted before combination with VSV.
[c] Assayed 48 hr after inoculation.

the primary target for uv radiation, failure of excessively irradiated SFV to modify the cultural behavior of DRK_3 cells suggests that a functional viral genome is required. Because cells inoculated with SFV inactivated by lower dosages of uv light became altered in the absence of detectable synthesis of viral DNA or major antigens, this required genome expression appears to occur early in the SFV replicative cycle.

Our experiments show that facilitation of VSV occurs in DRK_3 cultures infected simultaneously with SFV and VSV. Originally it was demonstrated that replication of VSV is facilitated in these cells under conditions in which replication of SFV was complete before superinfection with VSV (5). The present study indicates that concomitant replication of SFV, which did occur in the cultures inoculated with nonirradiated virus, does not interfere with replication of VSV. Interestingly, replication of VSV is also facilitated in rabbit cornea cells inoculated simultaneously with VSV and vaccinia virus, a cytocidal poxvirus related to SFV (8). This suggests that facilitation of unrelated VSV in certain kinds of mammalian cells may be a common property of poxviruses. The observed facilitation of VSV in DRK_3 cultures treated with partially uv-inactivated SFV, but not with SFV diluted to give a comparably low input multiplicity of infection, suggests that this cellular modification is also induced in the absence of complete SFV replication. Furthermore, lack of infectious VSV production in cultures treated simultaneously with excessively irradiated SFV indicates that facilitation requires a functional SFV genome. Whether a relationship exists between early SFV-induced modifications that alter the cultural behavior of DRK_3 cells and their ability to support replication of VSV will require further investigation.

Summary. When treated with uv radiation at dosages that abolished infectivity and prevented synthesis of detectable viral DNA or antigens, SFV remained able to modify the cultural behavior of DRK_3 cells and to make them susceptible to unrelated VSV. These cellular modifications were not induced, however, when the input SFV was excessively irradiated. The results suggest that a functional SFV genome is required to alter the DRK_3 cells and that early gene functions of SFV, before synthesis of viral DNA or major antigens, are involved in the modification process.

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