# Isolation of AGMK Cells Partially Resistant to SV40: Identification of the Resistant Step (36863)

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Infection of African green-monkey kidney (AGMK) cell cultures by SV40 usually results in viral reproduction and lysis of all the cells in the culture (1). In some cases, a minority of cells survives exposure to SV40 and gives rise to chronically infected cultures. Eventually some chronically infected cultures become transformed (2–6) and are resistant to superinfection by SV40 virions (3–8). In some cases transformed cells have been found susceptible to infection by SV40 DNA (8, 9). The mechanism by which AGMK cells become chronically infected and transformed by SV40 rather than lysed is unclear. However, it appears that the development of partial resistance to SV40 is a usual prerequisite (10). In order to better understand the nature of this partial resistance, we have studied the effect of viral specific anti-serum on chronically infected cultures and have characterized virus-free clones of cells isolated from chronically infected cultures. The step during the infectious process at which resistance occurs has been identified in one clone.

*Materials and Methods. Culure and cloning of cells.* The AH line (11) of AGMK cells in the 77th to 105th subculture was used for these studies. The medium used for routine culture was Eagle's basal medium with 10% fetal calf serum (FCS). Medium was changed as needed, usually twice weekly. Cells were subcultured when confluent. The pH of the medium was controlled by the use of 5% $CO_2$. For cloning, cells were dispersed with 0.25% trypsin and 0.05 ml aliquots of diluted cell suspension were placed in 35 mm petri dishes in medium containing rabbit anti-SV40 serum at 1:100 dilution and incubated at 37° in an atmosphere of 5% $CO_2$. Microscopic examination confirmed the initial presence of only one cell in those plates which subsequently grew out colonies which were considered clones.

*Virus.* Uncloned SV40 strain VA 45-54 and recently cloned SV40-W (12) were propagated by inoculating AH cells with the virus at a very low multiplicity of infection (MOI). When cell destruction was extensive, the cultures were frozen and thawed three times and spun at 1200 rpm. The supernatant fluids were stored at −60°. A virus stock was also collected from chronically infected AH cultures after 10 subcultures (see Results) and was designated SV40/AH.

*Virus assay.* Virus titers were determined by the plaque method as previously described (12). In addition, the simple presence or absence of virus in tissue-culture medium was determined by inoculating 0.1 ml of the fluid into tissue-culture tubes of AH cells. The cultures were observed for SV40 cytopathic effect (CPE) for a period of not less than 4 weeks.

*Extraction and assay of infectious SV40 DNA.* Virion pools were concentrated with polyethylene glycol and purified by equilibrium gradient CsCl sedimentation by the method of Friedman and Haas (13). Viral DNA was extracted from the purified virions with sodium dodecyl sulfate and phenol and plaque-assayed with DEAE-dextran as described by Pagano, McCutchan, and Vaheri (14). As reported previously no infectivity of SV40 DNA was found in the absence of DEAE-dextran (12).

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*Immune sera.* Rabbit anti-SV40 serum with a titer of 1:3760 as determined by virus neutralization tests was prepared by intravenous injection of virus together with intramuscular injection of virus mixed with Freund's incomplete adjuvant. Goat anti-SV40 serum with a titer of 1:320 was obtained from Flow Laboratories. Anti-serum against SV40 intranuclear T antigen was obtained from hamsters bearing tumors induced by the injection of virus-free SV40-transformed hamster cells. A fluorescent conjugate of rabbit anti-hamster serum was obtained from Microbiological Associates.

*Immunofluorescent studies.* The fluorescent antibody test for intranuclear SV40 T antigen was done according to the method of Pope and Rowe (15). Cells for T antigen studies were grown on cover slips in flat-sided tubes and tested on the 5th day after seeding unless noted otherwise. A minimum of 1000 cells for each line were examined in each experiment. Negative controls were uninfected AGMK cells and positive controls were SV40-infected AGMK cells.

*Experiments and Results. Establishment of chronic infections in AGMK cells.* Confluent monolayers of the AH line of AGMK cells were exposed to an uncloned stock of SV40 virus at a low MOI (0.001 to 0.5) and observed for viral CPE and for the presence of surviving cells. The usual result was progressive CPE and complete lysis of the cells in 10–21 days. In some of the cultures, however, small colonies of cells remained on the surface of the culture vessel and continued to grow despite the complete destruction of the rest of the culture. Usually these cultures were well populated with cells by 6 weeks and could be trypsinized and subcultured. The resultant subcultures usually continued to produce $10^5$-$10^7$ plaque-forming units (PFU) of SV40 per ml for many passages. No SV40 CPE was observed when these cultures were passed at confluence. Similar results were also obtained when infection was initiated in the AH line of cells using SV40 DNA at a very low input multiplicity (approximately 0.0001 PFU/cell).

In order to test whether the generation of resistant cells was due to an early interaction between the cells and a variant form of the virus present in the uncloned SV40 population, cultures of AGMK cells were inoculated in parallel at an input MOI of 0.02 PFU per cell using stocks of uncloned SV40 VA 45-54, recently cloned SV40 45-54 (SV40-W), and virus SV40/AH recovered from AGMK cultures 10 passages after chronic infections were established using the uncloned VA 45-54 stock. Colonies of resistant cells which developed into chronically infected cultures were obtained with all three virus stocks and there was no significant difference in the number of colonies obtained with the different viruses (Table I).

TABLE I. Establishment of Chronic Infections in AGMK Cultures Infected with SV40.[a]

| Virus[b] | Cultures surviving / Cultures infected |
|---|---|
| SV40 VA 45-54 | 6/25 |
| SV40-W | 3/25 |
| SV40/AH | 2/25 |

[a] Stationary cultures of AH cells in 16 × 150 mm tubes were infected with various SV40 stocks at a multiplicity of infection of 0.02. The number of chronic infections established due to the regrowth of persisting colonies of cells was determined.

[b] SV40 VA 45-54 is an uncloned stock. SV40-W is a stock made from cloned SV40 VA 45-54. SV40/AH is virus recovered from an AH culture in the tenth passage after being chronically infected with SV40 VA 45-54.

These results show that if a variant form of the virus in the uncloned SV40 stock was responsible for inducing resistance, it was also present at approximately the same frequency in the recently cloned SV40-W. In addition there was no evidence for enrichment of such a hypothetical variant when virus was collected from the chronically infected cultures after the 10th passage and used in the same experiment (Table I).

*Production of infectious virus and T antigen.* Eight AGMK cultures surviving infection by SV40 were studied for 30 or more passages to determine the outcome of infection. Control uninfected AGMK cultures were subcultured in parallel. T antigen

TABLE II. Characteristics of AGMK Cells Surviving Infection by SV40.

| Culture[a] | % of cells with T antigen[b] at passage | | | | | SV40 titer[d] $Log_{10}$ PFU/ml |
|---|---|---|---|---|---|---|
| | 1 | 14 | 14[c] | 30 | 37 | |
| AH/SV40-1 | 9 | 17 | ND[e] | ND | 15 | 6.0 |
| AH/SV40-2 | 19 | 20 | ND | ND | 30 | 6.7 |
| AH/SV40-3 | 17 | 100 | ND | 100 | ND | 1.0 |
| AH/SV40-4 | 5 | 8 | 16 | 14 | ND | 5.6 |
| AH/SV40-5 | 12 | 16 | 30 | 100 | ND | 1.0 |
| AH/SV40-6 | 7 | 10 | 18 | 8 | ND | ND |
| AH/SV40-7 | 6 | 50 | ND | 100 | ND | 1.0 |
| AH/SV40-8 | 2 | 6 | 8 | ND | ND | ND |

[a] Cultures AH/SV40-1 and AH/SV40-2 resulted from the regrowth of surviving colonies following infection of confluent AH monolayers with SV40 DNA at an input multiplicity of approximately .001. Cultures AH/SV40-3 through AH/SV40-8 were similarly obtained following infection with intact virions at an input multiplicity of approximately 0.5.

[b] Except for the second set of determinations made at passage 14, all cultures tested at the fifth day after subculture.

[c] Tested the eighth day after subculture.

[d] Determined by titration of the culture fluid removed at passage 18.

[e] ND signifies not determined.

studies and tests for virus production were done on all 8 lines at passage 1, 14 and 30 or 37. All 8 cultures continued to yield infectious SV40 during the time of this study, but there was a marked variation in the quantity of virus produced when cultures were tested at the 18th passage (Table II). At the time of the first subculture, 2–19% of cells in the chronically infected cultures were positive for SV40 T antigen as determined by immunofluorescent studies. Presumably T antigen-positive cells were the productively infected cells in the chronically infected cultures (see later experiments). Three of the 8 lines converted in time to 100% T antigen-positive and showed less virus production than cultures with lower percentages of T antigen (Table I), suggesting a conversion to the transformed state. None of the cell lines spontaneously became T antigen-negative. At passage 14 a comparison was made between the percent of T antigen-positive cells in cultures tested 5 days after seeding when the cells were still replicating and duplicate cultures tested on the 8th day after seeding when confluence was established. Three of four replicating cultures of chronically infected cells appeared to have a lower percent of T antigen-positive cells than confluent cultures adding new evidence to support the hypothesis of others (2) that replicating AGMK cells are somewhat more resistant to SV40 than stationary cultures.

*Treatment of chronically infected cultures with anti-SV40 serum.* Carrier-state infections (what we have termed chronic infections) are the result of a productive infection by virus of a few cells in a larger exposed population. These few cells release viruses which then reinfect a few more cells continuing the cycle of infection at a low rate. Such chronic infections can be "cured" of detectable signs of the virus by the use of specific anti-viral serum. Virus must be reintroduced to reestablish chronic infection in a cured culture. Three SV40-infected cell lines were maintained for 7 passages in medium containing rabbit anti-SV40 serum at a dilution of 1:100. This dilution of serum was sufficient to inactivate all SV40 in the fluids of these cultures as shown by direct assay of the fluids for SV40. Immunofluorescent studies for SV40 T antigen and assays for infectious virus were performed on all three cultures immediately prior to the treatment with antiserum and five days after the treatment was terminated. Two of the three cultures (AH/SV40-2 and AH/SV40-4) were "cured"

TABLE III. Effect of Anti-SV40 Serum on the Production of T Antigen and Infectious SV40 in Chronically Infected Cultures.[a]

| Culture | % of cells with T antigen | | SV40 titer $log_{10}$ PFU/ml | |
|---|---|---|---|---|
| | Before | After | Before | After |
| AH/SV40-2 | 9 | 0 | 6.7 | none |
| AH/SV40-3 | 100 | 100 | 1.1 | none |
| AH/SV40-4 | 7 | 0 | 5.6 | none |
| AH control | 0 | 0 | none | none |

[a] The cultures were maintained in rabbit anti-SV40 serum at a dilution of 1:100 from passage 19 to 26 and tested for T positive cells and infectious virus immediately before the antiserum treatment and 5 and 21 days after the exposure to anti-serum was terminated.

of the chronic infection by the antiserum treatment as determined by absence of T antigen and infectious virus. These cultures remained "cured" when tested again after 3 subcultures in the absence of antiserum (Table III). Reexposure of the lines to SV40 virus at the 3rd passage after curing resulted in the reestablishment of chronic infections. The foregoing results taken as a whole suggest a carrier-state infection as described earlier in lines AH/SV40-2 and AH/SV40-4. In contrast, the presence of T antigen-positive cells in one culture (AH/SV40-3) was not affected by anti-SV40 serum despite the fact that virus was no longer present in the culture after exposure to antiserum. This finding shows that the T antigen in these cells is not dependent on the presence of infectious extracellular virus and adds new evidence to support the assumption that 100% T positive AGMK cultures consist of transformed cells.

*Cloning of cells from chronically infected cultures.* Three cell clones, designated resistant clones 1, 2, and 3 were derived from one of the chronically infected cultures, AH/SV40-4 (Table II) in the presence of rabbit anti-SV40 serum at a dilution of 1:100. These three clones were free of T antigen and infectious virus when tested at passages 1, 3, 7, 16 and 20. In addition to looking for T antigen and infectious virus in these resistant clones, we also looked for evidence of viral coat protein by means of immunofluorescent staining. All results were negative. Attempts to demonstrate the presence of infectious virus in the resistant clones using a technique of co-cultivation with susceptible AGMK cells (16) were also totally negative. Tests for tumoregenicity by inoculation of $10^7$ cells of one of the resistant clones into the cheekpouches of 8 week old hamsters also yielded negative results. Chronic infections did not reoccur spontaneously in these clones but could be reestablished only by the addition of virus. All the clones showed partial resistance to SV40 when reinfected at the third passage with either a high (100 PFU/cell) or a low (0.01 PFU/cell) MOI. Viral CPE in the reinfected cultures was slight (using the low MOI) to moderate (with the high MOI) and all the cultures survived and formed chronic infections. Control AGMK cultures inoculated in parallel showed progressive SV40 CPE and eventually complete lysis. T antigen studies were done on resistant clones 1, 2, and 3 at the 3rd passage after reinfection. These cultures contained 8%, 2.4%, and 0.04% T positive cells, respectively. All three cultures contained infectious virus as determined by direct assay at passage 3. One of these virus-free clones was again tested for resistance to SV40 virons at the 16th passage (see later experiments and Tables IV and V) and found to be stably resistant. The isolation of virus-free clones of AGMK cells from chronically infected cultures and the demonstration that these cells maintain cellular resistance to SV40 virus for many passages in the absence of the virus is reported for the first time here.

*Infection of a partially resistant clone with SV40 DNA and SV40 virions.* Confluent cultures of resistant clone 2 at the 20th passage

TABLE IV. Production of T Antigen 48 Hr After Exposure of Resistant Clone 2 and Parental Line to SV40 Virions and DNA.[a]

| Cells | % of cells with T antigen after exposure to SV40 | | | |
|---|---|---|---|---|
| | Virions[b] | | | |
| | MOI = 100 | MOI = 1 | MOI = 0.1 | DNA[c] MOI = 0.1 |
| Resistant clone 2 | 29% | 0.5% | 0.04% | 1.1% |
| Parental AH line | 90% | 11.2% | 1.6% | 1.8% |

[a] Cultures of resistant clone 2 (passage 20) and the parental AH line were infected 5 days after subculture, when the monolayers had reached confluence.

[b] After exposure to virus during the 2 hr adsorption period, the cultures were washed three times with Hanks' solution and incubated at 37°. The medium contained 20 $\mu$g/ml of cytosine arabinoside in order to prevent multiple cycles of infection.

[c] After the cultures were washed twice with PBS ($Ca^{++}$- and $Mg^{++}$-free), SV40 DNA in PBS containing 1 mg/ml DEAE-dextran, M. W. 2 $\times$ $10^6$, was added for 30 min after which the cultures were washed twice with PBS and incubated in medium at 37°.

and of the parental AGMK line were exposed to SV40 DNA and SV40 virions at a high and low MOI to further study the nature of the resistance. The production of T antigen and infectious virus was then determined. Forty-eight hours after exposure to a low MOI (0.1 PFU/cell) the parental line had more than 40 times as many T positive cells as the resistant clone. In sharp contrast, when the resistant clone and the parental line were infected with SV40 DNA at the same MOI, there was at most only a two-fold difference in the numbers of T positive cells (Table IV). Similarly after exposure to a low MOI (0.1 PFU/cell), the production of infectious virus 72 hr after infection was $10^2$ to $10^4$ greater in the parental AH line than in the resistant clone when these cell lines were infected with intact virions (Table V). Moreover, when the dose of SV40 virions was increased or "crowded" cell cultures were employed, the differences between clonal and parent line, although smaller, were still apparent. In contrast, when the lines were infected with SV40 DNA, only a slight difference in production of infectious virus was found (Table V). Taken as a whole these results indicate that the barrier to infection in the resistant clone is at or prior to the step of viral uncoating.

*Discussion.* These findings are relevant to several problems concerned in the interactions that may occur between SV40 and host cells. It has been suggested that partial resistance to SV40 in AGMK cells is a result of an early virus–cell interaction (10). If this is indeed the origin of the cellular resistance, then the initial infection of the cells by the virus would appear to be an abortive infection or infection by a defective virus, since we have shown that cellular resistance is maintained through many cell passages in the absence of T antigen production and detectable evidence of infectious virus. Moreover, chronically infected cultures could be "cured" by anti-serum and the partially resistant cell clones isolated from a chronically infected culture in anti-serum remained virus-free and maintained resistance to SV40 for over 16 passages in tissue culture. To reestablish chronic infections in the "cured" cultures or in the virus-free clones, reinfection of the cultures was necessary. Presumably, reinfection with SV40 would also be needed for eventual transformation of these partially resistant T negative cultures.

The results just summarized as well as our failure to obtain any direct evidence that all or part of the SV40 virion persists in the partially resistant cells make it premature in our opinion to discard the simpler hypothesis that the observed resistance in some AGMK cells depends upon prior spontaneous somatic mutations which have given rise to cells partially resistant to SV40 rather than upon an early virus–cell interaction. According to

TABLE V. Production of Infectious Virus 72 Hr After Exposure of Resistant Clone 2 and Parental Line to SV40 Virions and DNA.

| Cells[a] | SV40 yield PFU/ml | | | |
|---|---|---|---|---|
| | Virion infection[b] | | | |
| | MOI = 100 | MOI = 1 | MOI = 0.1 | DNA[c] MOI = 0.1 |
| I. Confluent | | | | |
| Resistant clone 2 | $2 \times 10^6$ | $2 \times 10^4$ | $2 \times 10^2$ | $6 \times 10^5$ |
| Parental line AH | $8 \times 10^7$ | $6 \times 10^6$ | $1 \times 10^6$ | $8 \times 10^5$ |
| II. Crowded | | | | |
| Resistant cline 2 | $2 \times 10^7$ | $9 \times 10^5$ | $1 \times 10^4$ | ND |
| Parental line AH | $3 \times 10^8$ | $4 \times 10^7$ | $3 \times 10^6$ | ND |

[a] Cultures of resistant clone 2 (passage 20) and the parental AH line were either infected 5 days after subculture when the cells were barely confluent or 12 days after subculture when the monolayers were densely crowded with cells.

[b] After exposure to virus during the 2 hr adsorption period, the cultures were washed three times with Hanks' solution and incubated at 37° in medium with goat anti-SV40 serum at a final dilution of 1:16 to neutralize virus not removed by washing. After 18 hr the cultures were again washed twice with Hanks' solution to remove the antibody. The cultures were fed with Eagle's medium containing 10% FCS and were harvested by freezing 72 hr after inoculation.

[c] Cultures were exposed to SV40 DNA and incubated as described in footnote *b*, Table IV. At 72 hr, specimens were frozen for subsequent determination of the SV40 titer.

this view the virus would then merely select for such partially resistant cells. However, before this hypothesis can be confidently accepted, a search for SV40 DNA, m RNA, and proteins other than T antigen and coat protein in the resistant monkey cells will be required, since it has very recently been reported (18) that after abortive transformation by SV40, mouse cells may appear phenotypically normal and fail to produce T antigen yet, nevertheless, contain SV40 DNA. In addition, it may be possible to demonstrate the existence of incomplete or defective virions in the resistant clones by fusing them with other resistant clones or with defective AGMK transformed lines and rescuing complete virus by complementation (17).

In the resistant clonal line studied we have identified the step in the infectious process where partial resistance occurs as being at or prior to viral uncoating. In these cells virus and T antigen production were comparable to that of the parental control AGMK line when SV40 DNA was used for infection. However, there was a great difference in both T antigen and virus production when SV40 virions were used, especially at a low MOI. It has previously been reported that 100% T positive SV40-transformed AGMK cells are also resistant to infection by SV40 but susceptible to infection by DNA (8, 9). The new finding reported here shows that cellular resistance to SV40 in 100% T negative AGMK cells can occur without transformation necessarily following and that this resistance is a stable heritable characteristic of the cells. Moreover, the finding reported here and by others that transformed cultures are derived from chronic infections (2–6) suggests that the partial resistance manifested by the chronically infected cells is a usual prerequisite for the special virus–cell interaction that causes transformation, if the transforming virus is not defective.

*Summary.* African green-monkey kidney cell clones partially resistant to SV40 have been isolated in viral-specific anti-serum from chronically infected cultures. These clones were virus-free and produced no T antigen. One clone was found to be partially resistant to infection with SV40 virions but susceptible to infection by SV40 DNA. Chronically infected cultures were cured of infection by treatment with SV40 anti-serum while main-

taining their partial resistance to SV40.

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