Analysis of Hamster Tumor Cell Lines Isolated from Single Colonies Soon After Induction by Polyoma Strains Differing in Transplant Antigen Production

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Tumor cell colonies, arising after the infection of hamster embryo cells with two strains of polyoma virus (PV) which differed in their capacity to induce the PV-specific transplant antigen, were isolated and studied for their transplant and tumor or "T" antigen content and growth characteristics in vitro and in vivo. Four of four tumor lines induced by the transplant immunogenic 3049 strain contained the transplant antigen, while only one of seven lines induced by the immunogenically deficient sp-D strain showed direct evidence for this antigen. T antigen was not detected by immunofluorescence in three tumor lines induced by the 3049 virus which contained the polyoma transplant antigen. No differences in growth capacity on plastic, in agar, or in the hamster subcutaneous tissue were apparent in spite of significant differences in transplant antigen content among the clones.

It was previously demonstrated (3, 7) and confirmed by Jarrett (8), that the large-plaque virus (lp-D) derived by Dulbecco from the LID-1 strain of polyoma virus (PV) failed to induce transplantation immunity in hamsters to tumor cells known to carry the PV-specific transplant antigen. It was postulated on the basis of a recent study (5) that both the large-plaque and small-plaque strains of PV isolated by Dulbecco (lp-D and sp-D) were defective in their capacity to induce the transplant antigen in hamster tumor cells either in vitro or in vivo. A study was carried out to compare: (i) the frequency at which the immunogenic 3049 and nonimmunogenic sp-D viruses induced the PV-specific transplant antigen in hamster tumor cell lines isolated from colonies transformed in vitro and (ii) the growth characteristics and tumor (T) antigen production (2) of tumor cells differing in transplant antigen activity.

MATERIALS AND METHODS

Cell lines and virus strains. Hamster embryo cells, tumor cell lines, culture media, and virus strains have been described in detail elsewhere (3-7).

Standard assay for transplant antigen in vivo. A

standard assay for transplant antigen, as reflected by the rejectability of tumor cells in immunized versus control animals, was adopted. Eight hamsters were immunized with 10^6 plaque-forming units (PFU) of the 3049/P1B₂ strain of PV, a dose known to produce a 10- to 100-fold increase in rejection rate of tumor cell lines induced by that virus strain. Tenfold dilutions of tumor cells were inoculated subcutaneously into four positions on the abdomen of each animal, and the presence or absence of tumors in these sites was observed for a period of 6 weeks. The tumor dose 50% (TD₅₀) was calculated by the method of Reed and Muench (11) and the transplantation resistance index (TRI) was calculated from the ratio of TD₅₀ in immunized over control hamsters.

Immunofluorescence test for "T" antigen. Cells to be stained were grown on cover slips for 1 to 3 days, rinsed in tris(hydroxymethyl)aminomethane buffer, air-dried, and fixed 2 to 3 min in acetone at intervals after initiation of the culture. The immunofluorescent system which was used consisted of first reacting the fixed cells with a polyoma specific anti-T antigen serum (13) kindly supplied by K. K. Takemoto of the National Institutes of Health. Fresh normal hamster serum was added at an equivalent dilution as the anti-T serum, as suggested previously (12). The specifically adsorbed anti-T antibody was detected with a goat anti-hamster γ -globulin serum labeled with fluorescein obtained from Hyland Laboratories, Los Angeles, Calif. A positive control consisted of a tumor cell line Py-T-54, also supplied by K. K. Takemoto, which

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TABLE 1. *Comparative growth in vivo and in vitro of cell lines isolated from individual colonies appearing after infection of hamster embryo cells with 3049 and sp-D strains of polyoma virus*

Cell line	Number	Cloning efficiency ^a		Agar/plastic ($\times 100$) ^b	Transplantability ^c (TD ₅₀)	Cell type ^b
		Plastic	Agar			
HTC166/sp-D	1	12.0	0.8	7.0	200	Transformed ^e
	2	9.0	2.0	22.0	800	Transformed
	3	1.5	0.6	40.0	800	Transformed
	4	2.2	0.5	22.0	200	Transformed
	5	3.6	0.02	0.6	200	Transformed
	6	2.5	0.003	0.1	63,000	Transformed
	8	ND ^f	ND	ND	23,000	Transformed
	9	Single cells	<0.00001	—	>100,000 ^g	Normal ^h
	10	Single cells	ND	—	ND	Normal
HTC168/3049	1	ND	ND	ND	ND	Normal
	2	6.5	0.35	5.4	630	Transformed
	3	0.8	0.4	5.0	960	Transformed
	4	Single cells	<0.00001	—	>100,000	Normal
	5	7.0	0.30	4.3	1,000	Transformed
	6	Single cells	<0.00001	—	>100,000	Normal
	8	Single cells	<0.00001	—	>100,000	Normal
	9	Single cells	<0.00001	—	>100,000	Normal
	10	1.0	0.18	18.0	9,000	Transformed
HTC91/3049		0.50	0.20	40.0	100	Transformed

^a Ability of cells to form colonies either on plastic dishes or in 0.34% agar and measured as percentage of cells inoculated.

^b Percentage of cells capable of growing in agar compared to those growing on plastic.

^c Ability to transplant subcutaneously in hamsters measured in tumor dose 50% (TD₅₀).

^d Morphological characteristics of cell lines after isolation of clones in tissue culture.

^e Cells showing decreased contact inhibition with haphazard, criss-crossed pattern of growth.

^f Not done.

^g No tumor takes seen at 100,000 cell dose.

^h Larger, epithelial cells with good contact inhibition.

showed excellent immunofluorescence in this test. Appropriate negative controls showed no nonspecific staining with normal hamster serum or cells or with the goat anti-hamster fluorescein-labeled serum alone.

RESULTS

Growth characteristics of cells infected by the 3049 and sp-D virus strains. Hamster embryo cells were infected in suspension with either the 3049 or sp-D viruses at a multiplicity of 100 PFU per cell. One-hundred thousand cells were planted in plastic petri dishes (60 $\times$ 15 mm, Falcon) in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 10% tryptose phosphate broth (TPB). After 14 to 18 days, colonies were apparent, many of which showed a criss-crossing cellular orientation suggesting transformation. Ten suggestive colonies were picked at random from each set of virus-infected cell cultures, passed at weekly intervals, and eventually tested for a variety of behavioral characteristics. Most cultures derived from these origi-

nal colonies were examined between the 6th and 20th passages after the original isolation. Eleven lines, of 20 initiated, showed loss of contact inhibition and could be identified as transformed. Seven lines maintained normal morphology throughout, and two lines were lost before testing could be accomplished.

Table 1 summarizes the results of experiments to test simultaneously the ability of cells to proliferate in two different cultural environments as well as in vivo. Cell suspensions were prepared with trypsin, counted, and dilutions containing known numbers of cells were planted in a medium (10) consisting of 10% FCS + 10% TPB with or without 0.34% purified agar (Difco). The same dilutions were also inoculated into five hamsters subcutaneously to test transplantability.

From these comparisons, several points are of interest. In general, cells were capable of growing progressively to produce visible clones on plastic more frequently than when suspended in agar. This suggested that the agar matrix suppressed

TABLE 2. Growth of 3049 and sp-D tumor cell lines in control and 3049-immunized hamsters as a test for the polyoma transplant antigen

Cell line	Passage	Transplantability		TRI ^a	Transplant antigen
		Immune	Control		
HTC166/sp-D					
No. 1	11	159 ^b	100	1.6	0 ^c
2	17	317	216	1.5	0
3	18	237	1,000	0.2	0
4	20	100	100	1.0	0
5	18	1,590	178	8.4	+
5	29	134	195	0.7	0
6	16	21,500	3,160	6.8	+
6	27	3,160	500	6.3	+
8	26	18,700	23,200	0.8	0
HTC168/3049					
No. 2	16	100,000	3,200	31.0	+++
3	15	10,000	631	16.0	++
3	28	3,200	187	17.0	++
5	17	7,600	1,000	7.6	+
5	28	>50,000	1,340	>37.0	+++
10	16	>100,000	3,900	>26.0	+++
HTC91/3049		25,000	590	42.0	+++

^a Transplant resistance index, ratio of immune to control TD_{50} .

^b TD_{50} , number of tumor cells required to produce a tumor in 50% of animals.

^c Arbitrary measure of the content of transplant antigen based on the magnitude of the TRI; 0 = < 4.0, + = 4 to 10, ++ = 10 to 20, +++ = 20 to 100, ++++ = > 100.

the growth of from 60 to 99.9% of cells capable of growing successfully on plastic.

A second point is the relationship between ability to grow in agar or on plastic with transplantability. It is apparent that cells incapable of cloning either on plastic or in agar were likewise nontransplantable and retained a normal morphology with little loss of contact inhibition. However, among the transplantable cell lines, the correlation between cloning efficiency on plastic or in agar and growth in vivo was poor. The capacity of a cell line to grow in the hamster cannot be predicted from a knowledge of that cell's cloning efficiency in agar or on plastic.

Finally, the above conclusions pertained to all tumor cell lines, irrespective of the strain of PV used to induce the neoplastic alteration. However, in spite of the similarity between the tumor cell lines induced by the 3049 and sp-D virus strains in respect to their capacity to grow under three distinct environmental conditions, significant differences were observed in the transplant antigen related to virus strain.

Differences in the rejectability of individual tumor cell lines induced by two virus strains. Assays for the PV-specific transplantation antigen were carried out by comparing the ability of a tumor

cell line to grow in 3049 virus-immunized and control hamsters.

Table 2 summarizes the results of these experiments. Four of four 3049-induced tumor cell lines (HTC168/3049) showed a significant difference in TD_{50} between immune and control groups, which was reflected in TRI values greater than ten.

The early passage of number 5 showed a low TRI level, which, on reassay at a later passage, was significantly elevated. Included as a control, was an independently isolated cell line (HTC 91) which was induced in vitro, contained the PV-specific transplant antigen, and demonstrated the level of TRI found with cell lines which had been in culture for several years.

In contrast to the enhanced rejectability of newly transformed 3049/P1B₂ tumor cells in hamsters immunized by a standard 3049/P1B₂ inoculum, only one out of seven sp-D induced tumor cell lines (number 6) showed a low but significant level of rejectability in two separate assays. A second line (number 5) was positive in the first test and clearly negative in the second conducted at a later passage. This finding might suggest that the PV transplant antigen was present in early passages in sufficient levels to allow recog-

TABLE 3. *Evidence that clones of an sp-D induced tumor cell line lack measurable polyoma transplant antigen^a*

HTC166/ sp-D/ #4	Transplantability		TRI	Transplant antigen
	Immune	Control		
Clone a	194	194	1.0	0
b	80	50	1.6	0
c	50	32	1.6	0
d	50	133	0.4	0
e	158	254	0.6	0
f	80	80	1.0	0

^a See Table 1 and 2 for explanatory footnotes.

TABLE 4. *Failure to induce measurable transplant antigen in an sp-D tumor cell line by super-infection with the nondefective 3049 strain of polyoma virus^a*

HTC166/sp-D/ #4	Passage	Transplantability		TRI	Trans- plant antigen
		Immune	Control		
3049 Clone ^b a	3	158	195	0.8	0
b	3	232	158	1.5	0
c	3	135	195	0.7	0
d	3	500	316	1.6	0
e	3	316	630	0.5	0
f	3	316	1,350	0.2	0
g	6	134	158	0.8	0
h	7	194	100	1.9	0
i	7	183	500	0.2	0
j	7	187	134	1.4	0
k	7	211	158	1.3	0
l	7	233	194	1.2	0

^a See Table 1 and 2 for explanatory footnotes.

^b HTC166/sp-D/ #4 cells infected with 200 PFU per cell of 3049/P1B₂ virus and planted sparsely in plastic dishes from which separate clones were picked at random for transplantation studies.

nition and rejection, but diminished on subsequent passages.

Absence of transplant antigen in clones derived from a single sp-D transformant. One possible explanation for the failure of immune hamsters to reject an sp-D induced tumor cell line, derived from a single transformed colony isolated shortly after infection (16 days), was the presence in the population of a small proportion of cells which lacked the transplant antigen, but had a high capacity to initiate tumor formation, thereby escaping the immune mechanisms of the 3049/P1B₂ inoculated animals. This was tested by selecting at random clones from the HTC166/sp-D/ #4 line, and testing their behavior in the transplantation antigen assay. Table 3 shows the

results of this experiment. There was some slight heterogeneity among the individual clones in regard to their transplantability in control hamsters, but the behavior in immunized hamsters followed quite closely that in the controls.

Twelve other clones were isolated from the HTC166/sp-D/ #4 line after its infection with the transplant immunogenic 3049/P1B₂ virus at a multiplicity of 200 PFU per cell. This was done in an attempt to convert it from a nonrejectable into a fully rejectable line. As seen in Table 4, 12 out of 12 clones isolated retained the characteristic nonrejectable status similar to the parental HTC166/sp-D/ #4 line as well as the six clones isolated from it. These data indicate that the individual members of the HTC166/sp-D/ #4 population were homogeneous in their ability to initiate tumor formation *in vivo* and to resist host immune mechanisms.

Evidence for the presence of the PV transplant antigen in an sp-D tumor cell line. The direct *in vivo* assay method, designed to test the immunosensitivity of tumor cells, failed to show an increased resistance to sp-D induced tumor lines in animals immunized with a transplant immunogenic virus strain 3049/P1B₂. Therefore, a second approach, reported previously (5), was studied.

Hamsters were inoculated with 3 million X-irradiated HTC166/sp-D/ #3 or #4 cells intraperitoneally on day 0. The animals were subsequently inoculated 2 weeks later with a dose of 3049/P1B₂ virus at a level insufficient to stimulate high levels of transplant resistance alone, but which would act as an anamnestic stimulus if the animals had been specifically immunized initially. Table 5 shows the results of this experiment.

The virus alone produced a small increase in the TD₅₀, whereas the number 3 line with and without 3049/P1B₂ showed a moderate 10- to 11-fold increase in resistance, suggesting a non-specific immunization. However, in the case of number 4, the cells alone increased the TD₅₀ sixfold, but with both tumor cell and virus, a significant transplant resistance state was produced. This suggested that number 4 contained the PV transplant antigen in sufficient quantity to presensitize hamsters to the antigen, after which the virus could then augment the resistance anamnastically.

Production of T antigen by strains of polyoma virus. The capacity of the 3049 and sp-D strains to induce T antigen synthesis in mouse and hamster cells was tested. Mouse embryo cells were planted in dishes on cover slips and the monolayers infected with each strain 24 hr later. The hamster embryo cells were infected in suspension at a multiplicity of 100 PFU per cell and the infected cells were then planted in dishes. Cover

TABLE 5. Indirect evidence for the presence of the polyoma transplant antigen in an sp-D induced tumor cell line^a

Type of treatment	Tumor cell challenge (HTC3/3049)	TRI ^b
No treatment.....	257 ^c	1.0
3049/PIB ₂ ^a	1,080	4.2
X-irradiated HTC166/sp-D/3.....	2,810	11.0
X-irradiated HTC166/sp-D/3 + 3049/PIB ₂	2,610	10.0
X-irradiated HTC166/sp-D/4.....	1,630	6.4
X-irradiated HTC166/sp-D/4 + 3049/PIB ₂	15,900	62.0

^a Groups of eight hamsters were injected intraperitoneally on day 0 with 3-million HTC166/sp-D/ #3 or #4 tumor cells X-irradiated with 5,000 R just prior to inoculation. Two weeks later, groups were inoculated with 10^{4.0} PFU of the 3049/PIB₂ virus, as indicated, and challenged with serial 10-fold dilutions of the HTC3/3049 tumor cell line on day 28.

^b Transplant resistance index. See Table 2, footnote a.

^c TD₅₀. See Table 2, footnote b.

slips were air-dried and acetone-fixed at 24 and 48 hr after infection, and were then stained for T antigen.

Both virus strains induced the T antigen with essentially equal frequency in the nuclei of both mouse and hamster embryo cells. In the case of hamster cells infected in suspension with equivalent numbers of infectious units, 78% of cells produced T antigen.

This demonstration that the virus strains were equally effective in inducing T antigen synthesis in hamster embryo cells, indicated that the sp-D strain, abnormal in its production of transplant antigen, was fully competent to induce the synthesis of a second product of the viral genome in host cells. Tumor cells transformed by both virus strains were then tested for their content of T antigen with the immunofluorescence test.

By using as a positive control the Py-T-54 clone line of PV-induced hamster tumor cells provided by K. K. Takemoto, it was clear that a variety of tumor cell lines induced by either 3049 or sp-D virus strains produced no T antigen detected by immunofluorescence, as summarized in Table 6. One line, HTC166/sp-D/1, showed a weakly positive reaction in a majority of the nuclei. Two lines, HTC168/3049/3 and HTC166/sp-D/4, were infected with the 3049 and sp-D virus strains at a multiplicity of 100 PFU per cell, fixed and stained 24 hr later. In the HTC166/sp-D/4 line infected with 3049 virus and in the

TABLE 6. Immunofluorescent detection of polyoma T antigen in polyoma-induced hamster tumor cell lines

Cells tested	Nuclear fluorescence ^a	Transplant antigen ^b
Py-T-54 ^c	+++ ^d	NT ^e
HTC168/3049/ #3 control.....	0	++
HTC168/3049/ #3 + 3049/PIB ₂ ^f	0	NT
HTC168/3049/ #3 + sp-D ^f	+++ ^g	NT
HTC166/sp-D/ #4 control.....	0	0
HTC166/sp-D/ #4 + 3049/PIB ₂	+++ ^g	NT
HTC166/sp-D/ #4 + sp-D.....	0	NT
HTC3/3049.....	0	+++
HTC168/3049/ #5.....	0	+++
HTC166/sp-D/ #1.....	+ ^h	0

^a Nuclear fluorescence resulting from reaction of polyoma specific anti-T antiserum with the T antigen in fixed cells, and detected with a fluorescein-labeled goat anti-hamster γ -globulin (Hyland).

^b Detected by using the in vivo tumor transplant rejection test. See Table 2 for explanation.

^c A polyoma-induced, T antigen-positive tumor cell line supplied by K. K. Takemoto.

^d The majority of cells stained brightly in test.

^e Not tested

^f Cells infected in suspension with 100 PFU of virus strains indicated, planted on cover slips in culture, fixed and stained 24 hr later.

^g A rare cell (1 in 1,000) showed bright nuclear fluorescence.

^h Majority of cells showed slight nuclear fluorescence.

HTC168/3049/3 infected with sp-D, a rare cell (1 in 1,000) showed a typical bright fluorescing nucleus. The positive nuclei, after acute viral superinfection, served as a further internal control to indicate that these two tumor cell lines indeed lacked the T antigen, or that its level was below that required to give a positive immunofluorescence test.

DISCUSSION

This study confirmed the previous observation (3, 5, 7) that 3049 induced tumor lines contained the PV-specific transplant antigen, but only a rare line induced by the D virus strains showed evidence of this antigen as measured by a quantitative, in vivo, tumor rejection assay. In the present experiments, transformants were isolated soon after the neoplastic event in vitro, and the presence of the transplant antigen was measured in vivo. The primary frequency of transplant antigen

induction was found to follow the general pattern seen before (5). Only one of seven sp-D induced tumor cell lines showed a definite rejectability in immunized hamsters, but the extent of rejection ($TRI = 6.3$ to 6.8) was of lower magnitude than that seen with the majority of 3049 tumor cells ($TRI > 20$).

Since the assay procedure for the presence of transplant antigen was carried out in hamsters immunized in a standard way, it can be assumed then that the tumor cells induced by the two virus strains must vary in the manner of expression of the transplant antigen. This argument would lead to the interpretation that the difference in the behavior of the D strain was related to the manner in which cell membrane alterations were produced, giving rise to tumor cells with functionally less transplant antigen. This, in turn, could be explained by one of the several mechanisms: (i) underproduction; (ii) aberrant, internalized location of the transplant antigen determinants; (iii) incomplete immunochemical identity between the antigens; or (iv) the tumor cells arose spontaneously with little direct help from the D-virus strains. In the absence of a direct method of assay, alternatives i, ii, and iii cannot be resolved. That neither the large-plaque nor the small-plaque strain of D virus immunized hamsters against homologous tumor cell lines (5, 7) was evidence against the third mechanism. In addition, the demonstration that an sp-D tumor cell line (number 4) could sensitize hamsters to the PV-specific transplant antigen, as shown in Table 5, would favor either of the first two alternatives and argue against the fourth. Attempts to measure directly the antigenic moiety are in progress.

From a genetic standpoint, it was of interest that one sp-D tumor line showed a modest increase in rejection rate in immunized hamsters, suggesting that this cell represented an intermediate between the other more poorly rejectable sp-D lines and the more highly rejectable 3049 lines. This could be explained by the partial expression or penetrance of the viral gene controlling this cell membrane alteration, which in turn may be controlled in part by the cell genome itself. A finding which favors the participation of the host cell in this defect is provided by recent work of Defendi et al. (1), who found that a large-plaque strain, also derived by Dulbecco, was capable of inducing the transplant antigen in mouse cells and of immunizing mice against its own tumor cells. This virus, kindly supplied by V. Defendi, was found to lack ability to immunize hamsters against a hamster tumor cell line containing the specific transplant antigen (*unpublished data*).

Further evidence implicating the host cell in viral transplant immunogenicity has been suggested (4). The 3049 strain grown in hamster embryo cells was significantly more capable of immunizing hamsters against a PV-induced, transplant antigen-positive hamster tumor line than the same virus strain grown in mouse embryo cells. Recent studies by Winocour (14), showing that a portion of host cell deoxyribonucleic acid may be included in the PV virion, indirectly supports the concept of host cell participation in the transplant antigen phenomenon.

Studies of tumor cell growth characteristics under a variety of conditions in vitro or in vivo failed to show a consistent pattern related to either the inducing virus strain or to the presence or absence of functional transplant antigen. It was found that transplantable lines always acquired the capacity to grow in 0.34% agar, but that the agar cloning efficiency was variable when related to the capacity to form clones on plastic surfaces. This indicated that the agar environment was relatively restrictive, allowing only a fraction of cells capable of cloning on plastic to form colonies in agar. In spite of the high correlation between growth in agar and in the subcutaneous tissues of the hamster, quantitative comparisons did not show a good correlation. For instance, as seen in Table 1, the HTC166/sp-D/#2 grew in agar 100-fold better than number 5, in spite of the fact that the relative capacity to grow in the hamster was reversed. This lack of direct correlation between agar growth potential and transplantability has been observed in a variety of other PV-induced tumor cell lines studied in this laboratory (*unpublished data*).

These findings suggest that the ability to grow in agar and in the hamster are controlled by different but related cell functions, one of which may be the transplant antigen.

The relationship between T antigen production and presence of transplant antigen was studied. Tumor cells induced by both virus strains were found to be negative for T antigen as measured by a sensitive indirect immunofluorescence test using an anti-T hamster antiserum and a fluorescein-labelled goat anti-hamster globulin serum.

A T antigen-positive tumor cell served as a convenient control. It is of interest that cell lines induced by the 3049 virus containing significant transplant antigen activity, as well as those induced by the sp-D strain lacking this activity, were negative for T antigen. Only one sp-D line showed a faint immunofluorescent reaction. The two virus strains were equally capable of inducing large quantities of T antigen during a cytolytic cycle in mouse embryo cells, as well as an abortive cycle in hamster embryo cells.

On the basis of a carefully controlled, indirect immunofluorescence test, it was found that T antigen could not be detected in several 3049-induced hamster tumor cell lines which were transplant antigen-positive as measured by their immunosensitivity in polyoma-immune animals. This finding is of added significance in view of the very obvious positive result with the Py-T-54 cell in the same experimental protocol. Furthermore, other studies (1, 9) indicate that the immunofluorescent detection of T antigen or antibody is as sensitive as, or more sensitive than, the complement-fixation test. Since both techniques depend on the amount of antigen present, small but physiologically active quantities could be missed in either case. Although of interest, this finding does not allow final conclusions to be drawn as to the relationship between transplant and T antigens. However, on the reasonable assumption that the failure to detect T antigen in transplant antigen-positive cells reflects at least a reduced quantity of T antigen, these results then indicate a lack of correlation between the degree of expression of these two viral functions.

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LITERATURE CITED

1. DEFENDI, V., B., EPHRUSSI, H. KOPROSKI, AND M. D. YOSHIDA, 1967. Properties of hybrids between polyoma-transformed and normal mouse cells. *Proc. Natl. Acad. Sci. U.S.* **57**:299-305.
2. HABEL, K., AND D. AXELROD. 1965. Continued viral influence in virus-free polyoma tumors, p. 126-141. *In* M. Pollard [ed.], *Perspectives in virology*, vol. 4. Harper and Row, New York.
3. HARE, J. D. 1964. Transplant immunity to polyoma virus induced tumors. I. Correlations with biological properties of virus strains. *Proc. Soc. Exptl. Biol. Med.* **115**:805-810.
4. HARE, J. D. 1964. Transplant immunity to polyoma virus induced tumors. II. Evidence for host-dependent immunogenic variation of polyoma virus. *Proc. Soc. Exptl. Biol. Med.* **117**:598-603.
5. HARE, J. D. 1967. Transplant immunity to polyoma virus induced tumor cells. IV. A polyoma strain defective in transplant antigen induction. *Virology* **31**:625-632.
6. HARE, J. D., AND J. C. CHAN. 1966. Antigenic variation among polyoma viruses from different sources demonstrated by plaque neutralization. *Virology* **30**:62-73.
7. HARE, J. D., AND T. GODAL. 1965. Transplant immunity to polyoma virus induced tumors. III. Evidence for heterogeneity among transplant antigens *in vivo*. *Proc. Soc. Exptl. Biol. Med.* **118**:632-636.
8. JARRETT, O. 1966. Different transplantation antigens in BHK 21 cells transformed by four strains of polyoma virus. *Virology* **30**:744-747.
9. LEWIS, A. M., JR., W. H. WIESE, AND W. P. ROWE. 1967. The presence of antibodies in human serum to early (T) adenovirus antigen. *Proc. Natl. Acad. Sci. U.S.* **57**:622-629.
10. MACPHERSON, I., AND L. MONTAGNIER. 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology* **23**:291-294.
11. REED, L. J., AND H. MUENCH. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**:493-497.
12. TAKEMOTO, K. K., R. A. MALMGREN, AND K. HABEL. 1966. Heat-labile serum factor required for immunofluorescence of polyoma tumor antigens. *Science* **153**:1122-1123.
13. TAKEMOTO, K. K., R. A. MALMGREN, AND K. HABEL. 1966. Immunofluorescent demonstration of polyoma tumor antigen in lytic infection of mouse embryo cells. *Virology* **28**:485-488.
14. WINOCOUR, E. 1967. On the apparent homology between DNA from polyoma virus and normal mouse synthetic RNA. *Virology* **31**:15-28.