Immunofluorescent Studies of Mouse and Hamster Cell Surface Antigens Induced by Polyoma Virus[1]

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SUMMARY—The surface antigens of mouse and hamster polyoma-induced tumor cells and of mouse cells in the lytic cycle of polyoma virus infection were studied with the indirect fluorescent-antibody technique. Sera of mice immunized with polyoma virus or polyoma tumor cells were examined. Reactions were observed between the various sera and polyoma-transformed mouse and hamster cells or mouse cells in the lytic cycle. No reaction was noted with normal mouse embryo cells, simian virus 40-transformed mouse or hamster cells, or with mouse cells infected with herpesvirus. In addition, control sera from mice immunized with simian virus 40, a methylcholanthrene-induced tumor, or a mammary tumor failed to react with the polyoma-transformed cells.—J Nat Cancer Inst 40: 263–268, 1968.

VIRUS-INDUCED neoplasms in mice and hamsters have been shown by several different immunologic methods to contain new antigens specific for tumors caused by the same agent. These tests included complement fixation (*1–3*), immunofluorescence (*4–9*), and transplantation immunity (*10–12*). The evidence suggests that the antigens measured by the complement fixation and immunofluorescence tests are the same, but are apparently different from the transplantation immunity antigens. This concept is based on the observations that cross reactions occur between mouse and hamster polyoma tumors in the complement fixation and immunofluorescence tests (*3, 6*) but not in the transplantation immunity test (*13*).

Of great practical importance is the need for an *in vitro* method for measurement of transplantation immunity and for study of the tumor antigens associated with this type of immunity. Transplantation immunity to the polyoma virus tumors seems to be mediated by lymphocytes (*14*). However, many recent studies of antibody to the cell surface antigen by immunofluorescence (*7, 8, 15*) or colony inhibition (*13*) techniques suggest that the cell surface antigen may be an indicator of, or involved in, tumor transplantation immunity. Consequently, as part of a general effort to elucidate the factors involved in transplantation immunity, the surface antigens of mouse and hamster polyoma tumor cells were studied by use of sera prepared in various ways.

MATERIALS AND METHODS

Transformed cell lines.—The following lines were used:

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PY-1923 tissue culture cells derived from a transplantable polyoma virus-induced fibrosarcoma in a C57BL mouse and carried in culture for 1 year (*6*).

PY-T-54 cell line derived from a transplantable polyoma virus-induced fibrosarcoma in a Syrian hamster and carried in culture for 1½ years (*6*).

Simian virus 40 (SV40) hamster tissue-culture cell line originally derived from a transplantable SV40-induced hamster fibrosarcoma and carried in culture for 3 years.

AL/N mouse cell line of SV40-transformed cells produced *in vitro* and carried for 3 months.

These lines were tested and found free of polyoma virus and SV40 by inoculation of concentrated cell extracts into mouse-embryo and African green monkey kidney cultures. In addition, they all possessed the "T" antigen of their respective virus. The PY-1923 (*14*) and PY-T-54 cells, as well as polyoma tumor 695 (*14*), contained polyoma transplantation antigen.

Normal cells.—Cell cultures were prepared by exposure to trypsin of minced, NIH general-purpose, AL/N, C57BL, and BALB/c mouse embryos. The cell suspension thus prepared was grown as a monolayer culture in Eagle's medium with 10% fetal bovine serum.

Cells for lytic infection.—NIH general-purpose mouse-embryo cultures were infected with polyoma virus at a 100 to 1 multiplicity of infection. After 2 hours for virus adsorption, the cultures were treated with trypsin and suspended in spinner cultures. Under these conditions there was virtually no increase in cell number and the viability was better than 75% in all preparations, as tested by trypan-blue exclusion. Samples were tested at 4, 24, and 30 hours post infection. Other NIH mouse-embryo cultures were infected with small plaque-strain herpesvirus (*16*) at a 100 to 1 multiplicity. The cells were then prepared and tested in the same manner as the cells infected with polyoma virus.

Transplantable mouse polyoma tumors.—The C57BL polyoma tumor 695 (*17*) and a BALB/c salivary gland tumor induced by polyoma virus and carried in transplant for 1 year were dispersed by passage through a tissue press and then further reduced to a single cell preparation by exposure to 0.25% trypsin for half an hour.

Preparation of cells for surface fluorescence tests.—Tissue-culture cell suspensions were prepared for cell-surface immunofluorescence examination by treatment of the cells on glass with 0.25% trypsin for 20 minutes. The suspended cells were then centrifuged at 1000 × *g* for 10 minutes and resuspended in Eagle's medium containing 10% fetal calf serum. The cells were kept in suspension by means of a magnetic stirrer for 24 hours before being tested. This method was used because preliminary tests showed that prolonged exposure to trypsin or prompt tests after treatment seemed to reduce the number and intensity of the fluorescing cells.

Serum.—Collections were made from the heart of ether-anesthetized C57BL male mice which were: 1) bearing 2 × 2 cm or larger polyoma tumor 695; 2) transplanted with increasing numbers of the polyoma tumor cells (starting at 1×10^4 and increasing at 1-log increments every 2 weeks for at least 3 injections) and bled 2–3 weeks after the last tumor cell injection; 3) polyoma-virus immunized by 2 intraperitoneal injections of 0.2 ml containing 1×10^6 tissue-culture infectious doses (TCID) at 2-week intervals and bled 2 weeks after the last injection; 4) immunized with polyoma virus as described, challenged with 1×10^6 polyoma tumor 695 cells 2 weeks after the last dose of virus, and bled 2 months after tumor cell challenge; 5) normal; 6) SV40-immunized by 2 intraperitoneal injections of 0.2 ml containing $10^{5.94}$ TCID50/ml at 2-week intervals and bled 2 weeks after the last injection; 7) given subcutaneous injections of increasing doses of a cytosive preparation of a C3H mammary tumor at 2-week intervals for 4 months. The cell dose ranged from 5×10^6 to 40×10^6 and the sera were collected approximately 2 weeks after the last injection.[5]

Serum was also collected from C3H/HeN mice bearing 2 × 2 cm methylcholanthrene-induced Fibrosarcoma 55 and from C3H/HeN mice that had received transplants in the foot of MCA-induced Fibrosarcoma 55. The foot was amputated when the tumor was 3 × 3 mm. Serum was collected 2 weeks later.

Finally, serum was obtained from normal BALB/c mice, from BALB/c mice bearing a 2×2 cm or larger polyoma tumor, and from BALB/c mice inoculated with increasing numbers of syngeneic polyoma tumor cells, starting at 1×10^4 and increasing at 1-log increments every 2 weeks for

[5] We are indebted to Dr. Donald Morton of the Surgery Branch, NCI, for this serum.

at least 3 injections. Sera were collected 2–3 weeks after the last injection.

Immunofluorescence method.—Fifty-thousand tissue culture cells were pipetted into a 13 × 100 mm test tube and centrifuged. The supernatant was removed and the cells were suspended in 0.05 ml of the test serum. This suspension was incubated at room temperature for 30 minutes and then washed 2 times in phosphate-buffered saline, *p*H 7.4. The pellet was resuspended in horse anti-mouse γ-globulin labeled with fluorescein isothyocyanate [absorbed twice with bovine liver acetone powder 100 mg/ml (Progressive Laboratories, Baltimore, Md.) and then diluted 1 : 3] and incubated at room temperature for 30 minutes. The phosphate-buffered saline wash was repeated 3 times. The final pellet was resuspended in 25% glycerin in buffered saline and mounted beneath a coverslip. The preparation was examined with a Leitz ultraviolet microscope.

The fluorescence at the cell surface showed some variation in intensity but, in general, was either present or absent. When fluorescence was present the serum was considered positive and when absent it was considered negative. No positive reaction was observed in which less than 5% of the cells reacted, and in nearly all instances 20% or more of the cells were positive. Only fluorescence of the cell membrane, as previously described (*8*), was considered positive. The diffuse cytoplasmic fluorescence of dead cells was not counted.

RESULTS

The reactivity of the various antisera with the different cell lines is indicated in table 1. The antisera represent pooled sera from several animals. Since these were collected at different times, there were several samples for each type of serum. A freshly prepared cell suspension was used for each determination and the variation in reactivity of a serum seemed to be related to the reactivity of the particular cell preparation.

Serum "T" from C57BL mice bearing large, virus-free polyoma 695 tumors failed to react with most of the cell lines tested, although it did react once with PY-1923 and once with the BALB/c polyoma tumor cultures. On the other hand, serum "T" from mice immunized with 695 tumor cells frequently showed a relatively strong reaction with PY-1923 cells and with the polyoma-infected, NIH general-purpose, mouse embryo cells in the lytic cycle at 30 hours post virus infection. Sera of this type reacted once with hamster PY-T-54 cells and once with the BALB/c polyoma tumor cultures. The serum failed to react with cultures of the normal mouse cells, C57BL polyoma tumor 695, hamster or mouse SV40-transformed cells, or with the mouse cells infected with herpesvirus.

Antisera "V" prepared in mice against the polyoma virus reacted in about 23% of the tests with the virus-free PY-1923, once rather weakly with lytic polyoma-infected mouse embryo cells at 30 hours post infection, in 4 of 12 tests with the BALB/c polyoma tumor, and in 4 of 8 tests with the 695 tumor cells. Four of eleven tests with hamster PY-T-54 cultures also were positive. No reaction was detected with normal mouse embryo cells, the SV40-transformed mouse cells, or with the herpesvirus-infected mouse embryo cells.

Sera "T" and "V" from animals immunized to both the virus and the tumor reacted with the cell surface antigens to about the same extent as the sera against tumor cells alone. Normal C57BL mouse sera, on the other hand, failed to react with any cell line tested. In addition, sera from C57BL mice immunized with SV40 or with a C3H mammary tumor failed to react with the C57BL PY-1923 cultures.

Although the BALB/c mouse sera were not tested against all the cells, of interest was the relatively poor reactivity with the PY-1923 cultures. The sera "T" from animals with the BALB/c polyoma tumor was more reactive than the comparable C57BL sera with PY-1923 and lytic mouse embryo cultures. The sera "I" from mice immunized with the BALB/c tumor reacted in approximately half the tests against suspension of the same tumor cells or the C57BL mouse tumor 695. These sera failed to react, however, with cultures of PY-1923, hamster polyoma tumor, normal mouse embryo cells, SV40-transformed mouse cells, or herpesvirus-infected cells.

Normal and BALB/c mouse sera did not react with any of the cells. Sera from C3H/HeN mice with methylcholanthrene-induced Fibrosarcoma 55 or immunized with this tumor failed to react with PY-1923 cultures.

TABLE 1.—Reaction of mouse antipolyoma tumor and antiviral sera with the surface antigens of various transformed and normal mouse and hamster cells*

Antisera	C57BL† polyoma tumor 1923	C57BL‡ polyoma tumor 695	C57BL‡ embryo cells	BALB/c‡ polyoma tumor	BALB/c‡ embryo cells	NIH‡ embryo cells	AL/N‡ embryo cells	AL/N† SV40-transformed cells	Hamster† polyoma tumor PY-T-54	Hamster† SV40 tumor	Polyoma-infected‡ NIH mouse-embryo cells			Herpes-infected‡ NIH mouse-embryo cells		
											Hours post infection:			Hours post infection:		
											4	24	30	4	24	30
C57BL anti-695 "T"	1/9	0/6	0/3	1/9	0/3	0/6	—	—	0/8	—	0/1	0/3	0/1	0/3	0/3	0/3
Anti-695 "I"	12/23	0/2	0/1	1/3	0/1	0/15	0/1	0/1	1/8	0/2	0/1	0/2	1/1	0/1	0/1	0/1
Anti-"V"	5/22	4/8	0/4	4/12	0/4	0/12	0/4	0/4	4/11	—	0/1	0/3	1/1	0/4	0/4	0/4
Anti-695 "T" + "V"	7/14	1/4	0/2	2/6	0/2	0/4	0/6	0/6	1/5	0/1	0/1	0/3	0/1	0/4	0/4	0/4
C57BL anti-SV40 "V"	0/1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C57BL anti-C3H mammary tumor	0/1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C57BL normal	0/12	0/2	0/1	0/3	0/1	0/9	0/1	0/1	0/7	0/1	0/1	0/3	0/1	0/1	0/1	0/1
BALB/c anti-"T"	1/5	—	—	—	—	0/2	—	—	0/2	—	0/2	2/4	—	0/4	0/4	0/4
BALB/c anti-"I"	0/4	3/5	0/2	3/9	0/2	—	0/2	0/2	0/4	—	—	—	—	0/2	0/2	0/2
BALB/c normal	0/5	0/2	0/1	0/3	0/1	0/1	0/1	0/1	0/3	—	0/1	0/2	—	0/1	0/1	0/1
C3H anti-MCA 55 "T"	0/1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C3H anti-MCA 55 "I"	0/1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*Numerator = number of positive reactions; denominator = number of tests performed; "T" = sera from animals bearing tumor 2 cm or larger; "I" = sera from animals immunized with tumor cells; "V" = sera from animals immunized with polyoma virus.

†Established tissue-culture cell lines.

‡Solid tissue made up into a single cell suspension.

DISCUSSION

The specificity of the reactions observed seems to be high, as indicated by the lack of reactivity of the C57BL and BALB/c normal mouse sera with any of the cells. This is also evident from the failure of any of the positive antisera to react with the normal mouse embryo cells, with hamster or mouse SV40-transformed cells, or with the herpesvirus-infected cells.

Evidence presented by Kluchareva *et al.* (*15*) shows that the specific SV40 cell-surface antigen developed within 1 hour in human embryo cells and at 6 hours in hamster embryo cells infected with SV40 *in vitro*. In the present study, *in vitro* infection of mouse embryo cells with polyoma virus also demonstrated the development of the surface antigen at 24 and 30 hours, but not at 4 hours. The appearance of this antigen is of interest because it implies that the cell-surface antigenic structures are in a very dynamic state and the permanent alteration in cellular metabolism, including the formation of new cell surface antigen, is brought about by the virus. This seems to be true even though the cell is destined, in a relatively short time, to be destroyed by the viral cytopathogenic effect. Since presumably the same antigens are present on the transformed cells where cytopathogenic effect is not involved, it is evident that this cell surface change is not of itself important to the cytopathogenic process.

Although transplantation antigens of mouse and hamster polyoma tumors seem to be distinct (*13*), these tumors do share complement-fixing antigens (*3*). Unless this difference is due to greater sensitivity of the complement fixation test compared with the transplantation technique, it would suggest that different antigens are involved. The demonstration by Hellström and Sjögren (*13*) that antigens of polyoma tumors of mouse and hamster origin cross-react by the colony inhibition technique and the evidence presented here that by immunofluorescence a cross reaction is demonstrable suggest that the mouse and hamster do share surface antigens. However, the transplantation technique depends on the presence of cellular immunity, and this perhaps requires species-specific factors not required for the serum antibodies operative in the complement fixation, colony inhibition, and immunofluorescence methods. A point of interest in this regard in the present study is the evidence of a greater interspecies cross reaction with antisera prepared against the virus than with antisera against the tumor cells. Five of the six positive reactions observed between the mouse sera and the hamster polyoma tumor cells occurred with antiviral sera.

The reaction between sera from tumor-bearing animals and the mouse polyoma tumor cells is difficult to explain. Although it did not occur frequently, because it was seen indicates that all the antibody against the tumor cell surface was not being removed from the circulation by the tumor cells. This is in accord with the inability to passively transfer growth-inhibiting effect with "immune" sera in contrast to the successful transfer of transplantation immunity with "immune" lymphocytes. The demonstration of virus-specific surface antigen on polyoma virus-transformed cells is consistent with similar observations previously reported with the other papovavirus, SV40 (*8*).

ADDENDUM

After this manuscript was submitted for publication, similar results were described by Irlin in a paper, "Immunofluorescent Demonstration of a Specific Surface Antigen in Cells Infected or Transformed by Polyoma Virus," Virology 32: 725–728, 1967.

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