Transplant Immunity to Polyoma Virus-Induced Tumors. II. Evidence for Host-Dependent Immunogenic Variation of Polyoma Virus.* (29648)

J. Donald Hare† (Introduced by H. R. Morgan)
Louis A. Wehle Virus Research Laboratory, Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Transplant immunity against polyoma virus (PV)-induced tumor cells is elicited by inoculation of adult animals with live PV (1,2). This phenomenon can be explained by the production of a foreign tumor antigen in susceptible cells of the host by the inoculated virus(1). Active immunity, as manifested by resistance to transplanted PV tumor cells, is thereby stimulated. A basic question concerning this formulation is whether every cell in which the new, foreign antigen is produced is also neoplastic or whether cells can acquire the antigen without being altered in their growth characteristics. Assuming that both oncogenic and antigenic features of the PV-infected cells are determined by the viral DNA code, the question becomes, "How closely linked are the genes controlling these properties?"

Studies in this laboratory have shown that 2 strains of PV which differ in oncogenic potential are equally capable of producing transplant immunity(3). On the other hand, a third strain of PV does not cross-immunize against tumors induced by the first 2 strains in spite of the fact that it is oncogenic. This paper will describe a preparation of PV with significantly enhanced immunogenic potency when compared to its immediate parental virus and will present evidence that this may be an example of host-controlled modification.

Materials and methods. Virus strains. The 3049 and 210 strains of PV were received from Dr. Bernice Eddy and the large plaque (lp) strain was received from Dr. Renato Dulbecco. Stock virus was prepared in $P388D_1$ mouse lymphoma cells received from Dr. Allan Rabson, or occasionally in mouse embryo or baby mouse kidney cells(4). Plaque assays for infectious PV were performed as described previously(3).

Hamster tumor cell (HTC) lines. The HTC-3049-3 line was obtained from a tumor induced in a hamster with the 3049 virus and carried in tissue culture and by serial transplantation in adult hamsters as described previously(3).

Hamster embryo cells. Secondary cultures derived from hamster embryos were grown in Roux bottles fed with Eagle's "minimum essential" medium(5) containing 10% calf serum.

Anti-viral serum. The 3049 virus for immunization was propagated in baby mouse kidney cultures, clarified at 2,000 rpm for 10 minutes after freezing and thawing twice and then sedimented at 30,000 rpm for 2 hours. The sediment was resuspended in 1/100th the original volume and sedimented in a cesium chloride density gradient at 30,000 rpm for 18 hours. The band containing the heavy, complete particles was separated, mixed with complete Freund's adjuvant and 1 ml inoculated into each footpad of a New Zealand white rabbit. Booster injections without adjuvant were given 4 weeks after the initial injection and the animals bled at 6 weeks. Hemagglutinin-inhibiting antibody titer was approximately 40,000 units per ml.

Anti-tumor serum. The HTC-3049-3 tumor transplanted *in vivo* was prepared as a 50% homogenate, mixed with complete Freund's adjuvant and 1 ml inoculated into rabbits as described above. Booster injections without adjuvant were given at weekly intervals starting at 4 weeks postinoculation and the animals bled at 8 weeks.

Transplant immunity was demonstrated as previously described(3).

* Supported by grants from Nat. Cancer Inst., (CA-05206-03), and Nat. Inst. Allergy and Infect. Dis. (A1-04978-01), U.S.P.H.S., and from United Funds of Clayton and Alexandria Bay, N. Y.

† Supported by Research Career Development Award 5-K3-A1-13,959 from N.I.H., U.S.P.H.S.

TABLE I. Transplant Immunity Induced by Hamster Embryo Fibroblasts Infected with Polyoma Virus Strains.

Immunizing material	Pfu* per animal	HI antibody†	HTC-3049-3 tumor cell challenge dose 50,000	10,000	TD_{50}‖
Uninfected HEF‡	0	400	5/5§	5/5	<10,000
3049-Infected HEF	$10^{3.0}$	400	0/5	0/5	>50,000
"lp"-Infected HEF	$10^{3.0}$	400	4/5	3/5	~10,000

* Plaque-forming units.
† Hemagglutinin-inhibiting antibody, expressed as reciprocal of dilution per 0.25 ml.
‡ Hamster embryo fibroblasts—5 million cells per dose.
§ Tumor takes/No. injected.
‖ Tumor $Dose_{50}$, expressed as number of tumor cells injected subcutaneously, calculated by method of Reed and Muench(6).

Results. Immunity produced with infected hamster embryo cells. Previous studies(3) had shown that transplant immunity and HI antibody production in adult hamsters were dependent on virus dose. Since PV replicates poorly in hamsters, it seemed most likely that transplant immunity was dependent on the initial dose of infecting virus and thus directly related to the number of cells producing the tumor antigen. An experiment was performed to test the possibility that hamster cells infected with PV *in vitro* could acquire the tumor antigen. Hamster embryo cells were infected with approximately 1 plaque-forming unit per cell of the 3049 strain virus passed once in $P388D_1$ cells. The cultures were harvested by trypsinization after 4 days, cells washed with anti-PV rabbit antiserum (1%), resuspended to 5 million cells per ml and 1 ml inoculated into groups of hamsters intraperitoneally. Table I shows the results of this experiment. Hamster embryo cells infected with Dulbecco's "lp" virus as well as uninfected cells were inoculated as controls. The number of plaque-forming units of virus in the inoculum after freezing and thawing twice, as well as the hemagglutinin-inhibiting antibody level of a pool of serum from the hamsters in each group, were measured after the experiment. The animals were challenged with 50,000 and 10,000 HTC-3049-3 tumor cells 2 weeks after immunization. A striking degree of resistance was produced by the 3049 virus-infected hamster cell material which was out of proportion to the quantity of infectious virus present in the inoculum or the amount of HI antibody produced, based on our previous experience(3). The "lp" virus-infected cells showed much less immunizing capacity, supporting the findings already reported(3).

Enhanced immunogenicity of infected hamster cell preparations. The immunogenic capacity of 2 preparations of 3049 virus grown in $P388D_1$ mouse cells was then compared with hamster embryo cells infected 7 days with the same virus. Uninfected hamster cells induced no resistance to the tumor cell challenge (Table II). The mouse cell-propagated 3049 virus produced a low but significant resistance to challenge tumor cells which was of the same order of magnitude as we have regularly seen in this system. However, infected hamster cells, which contained at least 10-fold less virus, produced 5-fold more resistance than virus alone, as indicated by the dose of tumor cells required to produce a palpable tumor in 50% of the animals (TD_{50}). When the infected material was frozen and thawed 1 time and passed through a 0.45 μ Millipore® Filter, less virus was present and immunity approached that produced by the parental virus strain at 100-fold higher dose.

Evidence for virus-induced immunity. The results of the previous experiment pointed to either a highly immunogenic virus population or a soluble immunogenic antigen in the infected cell material. Two experiments were performed to differentiate between these alternatives. Table III shows the results of an experiment which demonstrated the ability of anti-PV serum to neutralize the immunogenic potency of infected cells. Hamster cells,

TABLE II. Enhanced Immunogenicity of Polyoma-Infected Hamster Embryo Fibroblasts.*

Immunizing material	Pfu per animal	HI antibody	HTC-3049-3 tumor cell challenge dose 60,000	10,000	TD_{50}
None	0	100	10/10	10/10	<10,000
Uninfected HEF	0	<100	6/6	6/6	<10,000
3049 Virus "early-passage"†	$10^{7.0}$	950	8/9	4/9	14,200
3049 Virus "late-passage"‡	$10^{8.5}$	920	10/10	4/10	13,500
3049-Infected HEF§	$10^{5.1}$	342	4/9	0/9	60,000
3049-Infected HEF filtered‖	$10^{4.3}$	<100	7/9	3/9	20,500

* See Table I for routine explanations.
† Passed 1 time in $P388D_1$ lymphoma cells, after receipt from Dr. B. E. Eddy.
‡ Passed 2 times in $P388D_1$ lymphoma cells, plaque picked and passed 4 more times in $P388D_1$ cells.
§ Infected with "early passage" 3049 virus and harvested 7 days after infection.
‖ Infected cells frozen and thawed 1 time, filtered through 0.45 mμ Millipore® Filter.

TABLE III. Neutralization of Immunogenic Capacity of Polyoma-Infected Hamster Embryo Cells with Polyoma Antiserum.*

Immunizing material	Pfu per animal	HI antibody	HTC-3049-3 tumor cell challenge dose 60,000	10,000	TD_{50}
None	0	200	10/10	10/10	<10,000
3049-Infected HEF	$10^{4.0}$	400	1/10	0/10	>>60,000
Idem + PV antiserum†	$<10^{2.0}$	214	10/10	7/10	<10,000
" + tumor antiserum†	$10^{4.3}$	303	5/10	0/10	60,000
" + PV antiserum + tumor antiserum†	$<10^{2.0}$	230	10/10	10/10	<10,000
" , U.V. treated‡	$10^{4.0}$	215	2/10	0/10	>>60,000

* See Table I for routine explanations.
† Antiserum treatment was 18 hr at 4°C with 1:100 dilution.
‡ Ultraviolet irradiation 40 cm for a germicidal lamp for 30 min.

infected for 7 days with 3049 virus, were trypsinized, washed, resuspended to 5 million cells per ml, frozen and thawed once, and treated with either rabbit anti-viral or anti-tumor cell serum at a concentration of 1:100 for 18 hours at 4°C. Another aliquot was irradiated with ultraviolet light for 30 minutes at a distance of 40 cm from a germicidal lamp before injection. Adult hamsters were then inoculated intraperitoneally with 1 ml of the appropriate preparations. It is apparent that antiserum to PV was most effective in reducing the immunogenic potency of this material. Ultraviolet irradiation was inadequate to destroy the virus or the immunogenic capacity. The reduction in immunizing potency by treatment with antitumor cell serum cannot be considered significant. It is of interest that this tumor antiserum was prepared with a virus-free, transplantable 3049 virus-induced tumor which did contain the specific PV tumor cell transplant antigen. In spite of this, the antiserum contained no antibody which either neutralized PV, as seen in this experiment, or inhibited PV hemagglutination, providing evidence against the presence of PV coat protein in the tumor cells containing no infectious virus.

A second experiment was designed to compare the immunogenic potency of infected hamster embryo cell homogenates in which the virus had been destroyed, with the same preparation treated with reagents which would partially destroy protein or lipoprotein cellular antigens and at the same time increase infectivity titers by destroying viral inhibitors. Table IV clearly demonstrates the importance of infectious virus for immunogenicity. When the immunizing material was treated with genetron or with RDE followed by trypsin, transplant resistance increased approximately 2-fold in parallel with infectivity. On the other hand, photodynamic inactivation of PV with toluidin blue(7) (6 μg per ml for 2 hours, 4 inches for Westinghouse Cool White fluorescent bulbs) signifi-

TABLE IV. Correlation Between Infectivity and Immunogenicity in a Polyoma-Infected Hamster Embryo Cell Preparation.*

Immunizing material	Pfu per animal	HI antibody	HTC-3049-3 tumor cell challenge dose 50,000	10,000	1,000	TD_{50}
None	0	<100	9/9	9/9	9/9	<1,000
3049 Virus mouse-grown†	$10^{9.8}$	2015	8/9	8/9	3/9	4,600
Idem in HEF homogenate‡	$10^{9.6}$	2260	8/8	8/8	1/8	4,800
3049-Infected HEF homogenate§	$10^{4.5}$	111	7/9	5/9	0/9	14,400
Idem: Genetron extracted‖	$10^{4.7}$	100	6/9	3/9	0/9	30,000
" : RDE + trypsin¶	$10^{4.6}$	100	7/9	3/9	1/9	23,000
" : Toluidine blue inactivated**	$<10^{1.0}$	<100	9/9	9/9	5/9	~1,000

* See Table I for routine explanations.
† Parental 3049 virus passed once in $P388D_1$ cells.
‡ Artificial mixture of parental 3049 virus in uninfected HEF homogenate.
§ 3049 virus infected HEF—frozen and thawed once before injection.
‖ Infected HEF homogenate was extracted once with equal volume of genetron.
¶ Infected HEF homogenate treated 1 hr with Receptor Destroying Enzyme 1:100 followed by 1 hr with trypsin 0.1% at 37°C.
** 6 μg/ml for 2 hr 4 inches from 2 Westinghouse fluorescent bulbs.

cantly reduced its immunizing capacity as well as its virus titer. In this experiment, parental 3049 virus, passed in mouse cells ($P388D_1$), was significantly less immunogenic. The immunizing capacity of the 3049 virus was not altered by mixing it with uninfected hamster embryo cell homogenate just prior to inoculation, ruling out a nonspecific, adjuvant-like action of hamster cells.

Dependence of altered immunogenicity on hamster cell passage. The enhanced immunogenicity of hamster embryo-grown virus could be explained by either a) host-controlled modification of its immunogenic behavior or b) selection of a virus strain, present in the parental, mouse-grown virus, with altered properties. An experiment was performed to discriminate between these 2 alternatives. If the enhanced immunogenicity after hamster cell passage were a host-controlled modification, a single growth cycle of hamster-grown virus ($3049/M_1/H_1$) in mouse cells ($3049/M_1/H_1/M_2$) would allow reversion of the virus property to that of the parental, mouse-grown virus ($3049/M_1$). However, if the character were genetically controlled, then passage back in mouse cells ($3049/M_1/H_1/M_2$) should enrich the high immunogenicity virus population. Table V shows the results of this experiment. It is clear that a single growth cycle of hamster virus ($3049/M_1/H_1$) in mouse embryo cells yielded virus ($3049/M_1/H_1/M_2$) which behaved more like the original, parental, mouse-grown strain ($3049/M_1$) than the hamster-grown virus. The virus obtained after a second passage in mouse cells was also highly oncogenic as measured by its ability to transform hamster embryo cells *in vitro* at a multiplicity of infection of 5. The parental mouse virus ($3049/M_1$) was less oncogenic, requiring a multiplicity of infection of 50 to produce only one quarter as many transformed colonies in the same test. The transformation assay used in this experiment was similar to that described by Stanners *et al*(8). Hamster embryo cells were infected in suspension and 100,000 cells planted in plastic Petri dishes in Eagle's medium with 10% calf serum. After 3 to 4 days' growth, the culture fluid was changed to Eagle's medium supplemented with 10% tryptose phosphate broth, 10% NCTC 109, with 2% bovine fetal serum. Transformed colonies grew rapidly in this medium, while normal cells either failed to grow or colonies of cells with normal morphology proliferated slowly. Cultures were fixed and stained after 10-14 days and evaluated for colony characteristics. Only those colonies showing typical criss-crossed, random cell orientation were scored as transformed.

Discussion. Many factors influence the ability of transplanted tumor cells to grow in a new host. One important factor is the innate ability of tumor cells to multiply *in vivo,* irrespective of immunological barriers.

TABLE V. Dependence of Enhanced Immunogenicity on Propagation in Hamster Embryo Cell Culture.*

Immunizing material	Pfu per animal	HTC-3049-3 tumor cell challenge 50,000	10,000	1,000	TD_{50}
None	0	8/8	7/8	5/8	~1,000
3049 Virus: mouse grown†	$10^{7.0}$	7/7	3/7	1/7	10,000
3049-Infected HEF homogenate‡					
1:0	$10^{4.0}$	2/7	0/7	0/7	>50,000
1:10	$10^{3.0}$	2/8	3/8	0/8	~50,000
3049-Infected HEF virus + 1 mouse cell passage§					
1:0	$10^{7.0}$	8/8	3/8	2/8	10,000
1:100	$10^{5.0}$	8/8	5/8	0/8	8,200

* See Table I for routine explanations.
† Parental 3049 virus passed once in $P388D_1$ mouse lymphoma cell.
‡ HEF infected 7 days with parental 3049 virus.
§ HEF-grown 3049 virus from (‡) passed once in mouse embryo cells.

A second factor is the development of specific humoral and cellular immune responses directed at destruction of the tumor cell transplants. The immunological nature of resistance to PV-induced tumors following infection of adult mice and hamsters with PV has been well established(1,2). The studies reported here demonstrate an interesting aspect of virus-induced transplant immunity. It has been possible to modify the degree of resistance to transplanted tumor cells developing in hamsters after PV inoculation by passing the immunizing virus once in hamster embryo cells. PV, strain 3049, which replicated in mouse cells ($3049/M_1$), produced less immunity to PV-induced hamster tumor cells than the same virus strain harvested after 4-7 days growth in hamster embryo cells ($3049/M_1/H_1$). Furthermore, when virus derived from hamster cells ($3049/M_1/H_1$) was allowed to proliferate in mouse cells again ($3049/M_1/H_1/M_2$), its immunogenic potency reverted to that of the parental virus ($3049/M_1$).

These results could be explained on the basis of one of the following postulates: a) a host-controlled modification of the virus during replication in hamster cells; b) selection of highly immunogenic virus particles from a mixed population with both high and low potency virus; and c) the increased immunogenicity of hamster cell-grown virus may be a result of merely reducing the dose of infectious virus. This could be explained if the higher dose of mouse-grown virus neoplastically transformed many more cells, thereby producing large quantities of transplant antigens. These could interfere with resistance either by saturating the mediators of transplant immunity, by producing a type of immunological tolerance or by stimulating circulating, humoral antibody rather than cellular immunity. This latter effect would be similar to tumor enhancement as visualized by Möller(9). Humoral antibody enhanced tumor growth by coating cells with autologous gamma globulin, thereby reducing their recognition by cellular mediators of resistance.

It is not possible on the basis of present data to decide between these alternatives. The dependence of immunogenic potency on host cell of origin superficially resembles host-controlled modification in bacteriophage systems(10). On the other hand, the third explanation satisfied this point equally well. However, it was shown in previous studies (3) that the immunogenic activity of the 3049 virus was lost when diluted to infectivity titers comparable to those shown to be highly immunogenic after hamster cell passage. Furthermore, dilution of the 3049 hamster-grown virus, passed once again in mouse cells ($3049/M_1/H_1/M_2$), did not increase its potency as would be expected if the third alternative were true (Table V).

The second alternative also seems less likely, since it requires that the poorly immunogenic virus not replicate effectively in hamster cells, while the immunogenic virus

must grow preferentially in hamster cells but not in mouse cells. The finding that 5 million hamster embryo cells infected 4-7 days with 3049 virus usually contained only 10^4 plaque-forming units of infectious virus indicated that even the immunogenic virus itself did not replicate effectively in hamster cells.

If the phenomenon reported here does represent the influence of host cell on a functional manifestation of the virus particle, the work of Habel and Axelrod(11) is of particular interest. They have shown that DNA extracted from PV propagated in mouse cells demonstrated greater homology with normal mouse cell DNA than with DNA from either normal or PV-induced neoplastic hamster cells. If the increase in immunogenicity described here can be shown to involve alterations of PV DNA by either selection or host-controlled modification, DNA homology studies will need to be interpreted from the viewpoint of the host cell in which the virus has replicated.

Further studies to correlate oncogenic and immunogenic potency of PV strains with the antigenic composition of hamster tumors induced by these strains should provide much better insight into the genetic and biochemical basis of oncogenesis.

Summary. The capacity of a polyoma virus strain to stimulate transplantation immunity in adult hamsters against tumor cells induced by the same strain was significantly increased by allowing the virus to proliferate in hamster embryo cells for 4-7 days. The high immunogenic capacity was lost by a further growth cycle in mouse cells, suggesting that host modification of some viral character may have taken place.

The author wishes to thank Mrs. Judith Vande and Miss Ruth Jennings for their excellent technical assistance.

1. Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 722.

2. Sjögren, H. O., Hellstrom, I., Klein, G., *Cancer Research,* 1961, v21, 329.

3. Hare, J. D., PROC. SOC. EXP. BIOL. AND MED., 1964, v115, 805.

4. Winocour, E., *Virology,* 1963, v19, 158.

5. Eagle, H., *Science,* 1959, v130, 432.

6. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

7. Hiatt, C. W., *Trans. N. Y. Acad. Sci. Ser. II,* 1960, v23, 66.

8. Stanners, C. P., Till, J. E., Siminovitch, L., *Virology,* 1963, v21, 448.

9. Möller, G., *J. Nat. Cancer Inst.,* 1963, v30, 1153.

10. Stent, G. S., in *Molecular Biology of Bacterial Viruses, Freeman,* San Francisco, 1963, p376.

11. Habel, K., Axelrod, D., in *Perspectives in Virology,* M. Pollard, Ed., Wiley, New York, 1964, in press.

Received July 15, 1964. P.S.E.B.M., 1964, v117.

7S Versus 19S Anamnestic Response in Rabbits. (29649)

YVES BOREL, MARTHE FAUCONNET AND PETER A. MIESCHER
Department of Medicine, New York University School of Medicine

While a number of soluble antigens have been shown to induce immunologic tolerance in newborn rabbits, it has been more difficult to induce tolerance with corpuscular antigens (1,2). As yet no tolerance has been achieved with heterologous erythrocytes in newborn rabbits. Rather, small doses of heterologous erythrocytes produced immunization(3).

In pursuing this problem we have found an unexpected pattern in the anamnestic response of newborn rabbits injected with large amounts of antigen. These animals, after having produced a preferentially 7S immune response, were shown to exhibit later in the course of the experiment a 19S anamnestic response. Such a pattern has not been described previously. This observation led us to investigate the pattern of immune response in newborn rabbits in relation to the dose of antigen and the route of its administration.

Material and method. Newborn rabbits from outbred white females were used