Transplant Immunity to Polyoma Virus Induced Tumors. III. Evidence for Heterogeneity Among Transplant Antigens *in vivo*.* (29924)

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

In a previous study(1), it was shown that Dulbecco's "large plaque" strain of polyoma virus (PV), here designated "lp-D," failed to immunize adult hamsters against tumor cells induced by a second large plaque strain, 3049, originally obtained from Dr. B. E. Eddy. These two strains were, however, indistinguishable in most characteristics including oncogenicity, serological behavior(2), high hemagglutinin production, and plaque morphology. On this basis, then, they differed only in their capacity to stimulate transplantation immunity, and thus provided a possible genetic marker with which to investigate the role of the viral genome in induction of the new tumor cell antigen(3,4). This paper will describe the results of studies designed to clarify the mechanisms by which the "lp-D" strain of PV not only failed to immunize against a tumor cell induced by the 3049 strain, but tended to enhance its growth.

Materials and methods. The propagation, characteristics, and source of the virus strains and hamster tumor cell (HTC) line 3049-3 have been described(1). The lp-D virus-induced tumor cell line, HTC-lp-57H, was isolated from a sarcoma induced in a newborn hamster in a manner identical with the HTC-3049-3 line(1). A tumor cell line, induced by the 3049 virus in hamster embryo tissue culture and carried in the same manner as the other cell lines, has been designated HTC-3049-TC10. Due to the inherent variability in transplantability of tumor cell lines from one experiment to the next, the studies reported here always included an appropriate, untreated control group. On this basis, comparison of tumor takes in 2 different experiments must be limited.

* Supported by grants from U.S.P.H.S. CA-07239 and CA-05206.

† Supported by U.S.P.H.S. Career Research Development Award K3-13,959.

Results. Failure of 2 virus strains to stimulate reciprocal cross immunity. From initial studies(1), it was clear that the 3049 virus could stimulate transplant immunity against a tumor cell line induced by itself, but that the Dulbecco lp strain (lp-D) consistently failed to protect hamsters against the same tumor cell. A number of lp-D virus-induced primary tumors were put into tissue culture in an attempt to produce tumor cell lines for transplantation studies. Only one line, HTC-lp-57H was sufficiently transplantable to compare with the HTC-3049-3 line.

Table I shows results of an experiment in which animals injected with either the 3049 or lp-D viruses, propagated in P388 D_1 mouse lymphoma cells, were challenged with tumor cells derived from each virus. The degree of transplant immunity produced in either case was low. There was a definite trend toward homologous resistance with no cross-resistance. The tumors were smaller in animals immunized with the homologous virus and larger with the heterologous virus. The mean tumor weights include animals without tumors considered as having a tumor weight of 0. It is apparent that tumor size in the animals immunized with the virus which originally induced the tumor was much smaller than the tumor size in animals immunized with the heterologous virus. Tumor growth in animals immunized with heterologous virus was somewhat better than that in control, untreated animals, a phenomenon suggestive of "tumor enhancement."

The results of this experiment indicated no cross-protection between the 2 virus strains and tumor cells induced by each. However, recent studies in this laboratory(5) have shown that the capacity of the 3049 virus to produce transplantation resistance was significantly increased after growth of the virus for 4 to 7 days in hamster embryo cells. To test the possibility that this method of propa-

TABLE I. Lack of Cross-Immunogenicity Between 2 Polyoma Virus Strains.

Immunizing virus	Pfu* per animal	HI† aby	Tumor cell challenge: HTC-3049-3 10,000 cells Tumor take	HTC-3049-3 10,000 cells Tumor wt	HTC-lp-57H 11,000 cells Tumor take	HTC-lp-57H 11,000 cells Tumor wt
				(mg)		(mg)
None	0	<400	5/6‡	581	5/6	315
3049§	$10^{6.5}$	3200	6/9	39	9/9	363
lp-D§	$10^{5.5}$	1480	9/9	1233	6/9	131

* Plaque forming units.
† Hemagglutinin-inhibiting antibody, geometric mean titer, expressed as the reciprocal of dilution per 0.25 ml.
‡ Tumor takes/No. injected.
§ Propagated in P388 D_1 mouse lymphoma cells.

gation might increase the ability of the lp-D virus to protect hamsters against a heterologous tumor cell, a second experiment was performed in which the transplant immunogenicity of the lp-D virus was compared with that of the 3049 virus, both after growth in hamster embryo cells. The results of this experiment are shown in Table II. A relatively low dose of the 3049 virus grown in hamster cells stimulated potent immunity against its own tumor cells, but tended to increase tumor takes with the lp-D virus-induced tumor cell. On the other hand, the lp-D virus failed to protect hamsters against its own tumor cell, but did increase the number of takes as well as size of the 3049-induced tumor. An even more interesting phenomenon was seen in animals given both virus preparations simultaneously. Here the development of homologous resistance was interfered with, as shown primarily in the case of the HTC-3049-3 tumor cell, while the lp-D virus-induced tumor was enhanced.

Enhancement of tumor cell growth by immunization with heterologous virus. A striking example of "tumor enhancement" occurred in an experiment in which the 3049 virus-induced tumor cells were used to challenge animals immunized with either 3049, Dulbecco's small plaque (sp-D) or lp-D virus strains. The challenge tumor cells utilized had been carried continuously *in vitro* for 90 subcultures and did not transplant readily. Table III shows the results of this experiment. Here the homologous virus, 3049, failed to protect against its own tumor cells perhaps indicating a loss of the 3049 virus-specific cell identity antigen. However, both the lp-D and sp-D virus strain significantly reduced the number of cells required to produce a tumor in 50% of the animals (TD_{50}). It is of interest that the latter 2 strains, derived originally from the same virus stock, brought about "enhancement," indicating that the sp-D strain behaved in a manner similar to the lp-D strain in relationship to a 3049-derived tumor cell line.

Enhanced tumor transplantability following immunization with tumor cells. The ability of several tumor cell lines, induced by

TABLE II. Lack of Cross-Immunogenicity Between 2 Polyoma Virus Strains.*

Immunizing virus†	Pfu per animal	HI aby	Tumor cell challenge: HTC-3049-3 60,000	HTC-3049-3 10,000	HTC-3049-3 TD_{50}‡	HTC-lp-57H 60,000	HTC-lp-57H 10,000	HTC-lp-57H TD_{50}
None	0	<100	8/8	5/8	~10,000	5/8	3/8	31,000
3049	$10^{4.98}$	1040	2/9	0/9	>60,000	8/9	6/9	<10,000
lp-D	$10^{4.56}$	400	9/9	9/9	<10,000	7/9	1/9	28,000
3049 + lp-D	$10^{4.82}$	800	6/9	5/9	~10,000	7/9	7/9	<10,000

* See Table I for routine explanations—1,2,3.
† Each virus was grown in hamster embryo cultures for 7 days, the cells harvested, adjusted to 5 million cells per ml, frozen and thawed once and 1 ml inoculated intraperitoneally 14 days prior to tumor cell challenge. The mixture of 3049 and lp-D was prepared by mixing equal parts of each cell suspension and 1 ml was inoculated intraperitoneally.
‡ Tumor $dose_{50}$—calculated by method of Reed and Muench(13).

TABLE III. Enhanced Tumor Transplantability in Hamsters Immunized with Heterologous Polyoma Virus Strains.*

Immunizing Virus†	Pfu per animal	HI aby	Tumor cell challenge—HTC-3049-3 91,000	9,100	910	TD_{50}
None	0	200	5/5	1/5	1/5	22,000
3049	$10^{7.0}$	951	4/5	2/5	0/5	19,000
lp-D	$10^{5.54}$	1050	5/5	5/5	5/5	<910
sp-D	$10^{6.72}$	800	5/5	4/4	3/4	<910

* See Table I and II.
† Each virus stock was propagated in P388 D_1 mouse lymphoma cells.

TABLE IV. Enhanced Tumor Transplantability in Hamsters Immunized with X-Irradiated Polyoma Virus-Induced Tumor Cells.*

	Tumor cell challenge: HTC-3049-3				HTC-lp-57H			
Immunizing material†	60,000	10,000	1000	TD_{50}	100,000	10,000	1000	TD_{50}
None	3/6	0/6	0/6	60,000	0/7	0/7	0/7	>100,000
HTC-3049-3 10^7 cells	4/7	4/7	4/7	<1,000	2/7	0/7	0/7	>100,000
HTC-3049-3 10^6 cells	6/6	6/6	4/6	<1,000	5/6	2/6	0/6	80,600
HTC-3049-TC10 10^7 cells	2/7	2/7	0/7	~60,000	1/7	0/7	0/7	>100,000
HTC-lp-57H 10^7 cells	3/6	1/6	0/6	60,000	0/7	0/7	0/7	>100,000

* See Tables I and II.
† Tumor cells grown in culture, X-irradiated with 5000 R just prior to intraperitoneal inoculation of the indicated number of cells 14 days prior to tumor cell challenge.

different PV strains, to stimulate transplant immunity against the HTC-3049-3 and lp-D-57H cell lines was then tested. The tumor cells were X-irradiated with 5000 R and 10^6 or 10^7 cells inoculated intraperitoneally 2 weeks before the animals were challenged simultaneously with 3049 and lp-D tumor cell lines. The results are shown in Table IV.

It is of interest that X-irradiated tumor cells failed to immunize against either lp-D or 3049 virus-induced tumor cells, but rather the 3049 virus-induced line enhanced its own growth as well as that of cells induced by the lp-D virus. In this experiment, only the HTC 3049-3 line produced enhancement, while 2 other lines, one induced by the lp-D virus *in vivo* and another by the 3049 virus in tissue culture, failed to affect the transplantation of either tumor cell line. In another experiment, however, not shown here, a second lp-D virus-induced tumor cell line (57-G), as well as the 3049-3 line, enhanced the same 2 challenge tumor cell lines, while one sp-D virus-induced tumor (51-I) did not alter their transplantability. In both experiments, the lower concentration of 3049-3 tumor cells, *i.e.*, 10^6 cells, produced a higher degree of enhancement than 10^7 cells.

Discussion. Two strains of PV which superficially resemble each other, 3049 and lp-D, failed to immunize hamsters against tumor cells induced by the other virus, but rather tended to enhance the growth of the heterologous cell. This reproducible behavioral distinction between 2 virus strains described here implies that this is related to the genetic apparatus of the virus, since it is expressed not only in the adult hamster during stimulation of transplant immunity, but also in the transplantable tumor cells used to test that immunity.

It is of importance to note that each virus strain behaved in a different way than the tumor cells induced by the same virus. The 3049 virus demonstrated the paradoxical activity most clearly. When grown in hamster embryo cells, this strain strongly interfered with the transplantability of a tumor cell induced by itself but increased the transplantability of a cell induced by the lp-D virus (Table II). This could be explained if the 2 viruses produced different cell identity antigens. However, when the same 3049 virus-induced tumor cell was X-irradiated and injected into hamsters, enhancement of both 3049 and lp-D tumor cells resulted.

Kaliss(6) and Møller(7) have shown that humoral antibody directed against a histocompatibility antigen could interfere with cell-mediated, tumor homograft rejection and that the interference was quite specific. A possible explanation for the findings reported here, based on this action of humoral antibody in tumor enhancement, could be visualized as follows. The immunizing virus might stimulate the synthesis of a transplantation antigen in cells of the adult hamster identical with the major identity antigen of the homologous tumor cell as postulated by Habel (8). The adult host would then respond with active cellular immunity directed against this antigen. In order to explain reciprocal enhancement, the virus must also cause the production of a second antigen, identical with the major antigen in tumor cells induced by the heterologous virus, but in a form or location which stimulated humoral antibody rather than cellular immunity. This circulating antibody to the heterologous tumor cell antigen could then interfere with cell-mediated immunity to bring about enhancement. Finally, enhancement of both homologous and heterologous tumor cells by either a 3049 or lp-D virus-induced tumor alone would indicate that both antigens were present in one cell line and that the predominant immunological response to the X-irradiated cells was circulating antibody to both antigens. It has not as yet been possible to demonstrate a humoral, tumor-enhancing factor in such sera in this laboratory.

A second possible explanation is related to a phenomenon described by Elkins(9). He showed that the immunogenicity of a weak transplant antigen was reduced in mice previously immunized with another more strongly immunogenic transplant antigen. In order for the suppression to be effective, it was required that the strong antigen be carried by the same cell as the weak antigen to which transplant immunity was being tested. Although a definitive explanation for this observation could not be given, Elkins felt that circulating antibody played no role. It is conceivable that in a similar fashion, the immunizing PV produced the homologous antigen to which the animal responded with transplant immunity, but that upon challenge with tumor cells containing a second antigen, the host-immune mechanism failed to recognize or to respond to the second stimulus, *i.e.*, the heterologous, challenge tumor cell, with the appropriate cell immunity. The resulting lack of growth restriction would resemble enhancement.

It is not possible on the basis of present data to choose between these 2 alternatives or to propose others. In either case, the data fit best with a hypothesis which requires the presence of 2 different cell identity antigens. The first hypothesis can be recognized as involving the efferent limb of the immunological response in which one type of positive response (antibody) interferes with a second type of response (cellular immunity). The second hypothesis requires that there be a failure in the afferent limb such that the virus-immunized host cannot recognize the heterologous antigen carried on the challenge tumor cells and thus fails to reject the cells identified by the presence of this antigen. It is of primary interest however, that either resistance or enhancement of tumor growth can be produced in one animal by the action of a tumor virus. Further studies are in progress. Finally, the 3049 and lp-D strains could be considered as mutants differing not in the structural genes specifying 2 antigens but rather in genes determining the form, quantity or location in which the 2 antigens are incorporated into the cells of the adult hamster to which it makes a specific immunological response. Recently, Ting (personal communication) has confirmed the finding of an immunogenic distinction between the SE 3049 and large plaque viruses in another species, the mouse. Furthermore, when the 3049 virus was propagated in a susceptible, lp-D induced mouse tumor cell, 4198, the progeny virus was now capable of stimulating transplant immunity in mice against the lp-D tumor cell. This alteration in immunogenic behavior is suggestive of "marker rescue." This approach may help to clarify the role of viral genetic material in induction of the transplant antigens. Studies of the complement-fixing antigens in different tumors and antibodies in hamsters which carry those tumors(10,11,12) may help to determine whether the CF and cell identity

antigens are one in the same substance.

Summary. The ability of 2 polyoma virus strains, 3049 and lp-D, to produce transplant immunity in adult hamsters against tumor cells induced by both strains was studied. The 3049 virus immunized hamsters against tumor cells produced by itself, but tended to enhance the growth of lp-D virus-induced tumor cells. On the other hand, the lp-D virus produced little or no immunity against its own tumor cell, but enhanced the growth of 3049 virus-induced cells. X-irradiated tumor cells induced by the 3049 virus, when given to adult hamsters, enhanced the growth of both 3049 and lp-D tumors. Possible explanations for these paradoxical results, based on the presence of at least 2 transplant antigens, were discussed.

The authors wish to acknowledge the excellent technical assistance of Mrs. Judith Vande and Miss Ruth Jennings.

1. Hare, J. D., Proc. Soc. Exp. Biol. and Med., 1964, v115, 805.

2. Hare, J. D., Morgan, H. R., Virology, 1963, v19, 105.

3. Habel, H., Proc. Soc. Exp. Biol. and Med., 1961, v106, 722.

4. Sjogren, H. O., Hellstrom, I., Klein, G., Cancer Research, 1961, v21, 329.

5. Hare, J. D., Proc. Soc. Exp. Biol. and Med., 1946, v117, 598.

6. Kaliss, N., Ann. N. Y. Acad. Sci., 1962, v101, 64.

7. Møller, G., J. Nat. Cancer Inst., 1963, v30, 1153.

8. Habel, K., J. Exp. Med., 1962, v115, 181.

9. Elkins, W. L., J. Immunol., 1964, v92, 275.

10. Huebner, R. J., Rowe, W. P., Turner, H. C., Lane, W. T., Proc. Nat. Acad. Sci., 1963, v50, 379.

11. Habel, K., Axelrod, D., Perspectives in Virology, M. Pollard, Ed., 1964.

12. Defendi, V., Ephrussi, B., Koprowski, H., Nature (London), 1964, v203, 495.

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

Received September 17, 1964. P.S.E.B.M., 1965, v118.

Fatty Acid Metabolism by *in vivo* Labeled Adipose Tissue in Essential Fatty Acid Deficient Rats.* (29925)

OLGA STEIN AND YECHEZKIEL STEIN

Departments of Experimental Medicine and Cancer Research, Biochemistry and Department of Medicine "B", Hebrew University-Hadassah Medical School, Jerusalem, Israel

In a previous study it was shown that epididymal fat pads of rats esterify and metabolize palmitic and linoleic acid at a similar rate(1). These results were obtained in normally fed, growing rats, in which the epididymal fat pads were labeled selectively with radioactive fatty acids, using the *in vivo* incubation technique(2). The present communication deals with the comparative uptake and dissimilation of palmitic and linoleic acid in epididymal fat pads of essential fatty acid (EFA) deficient rats.

Materials and methods. Male albino rats of the Hebrew University strain were kept on a fat free diet(3) from weaning. Since hydrogenated oil was not added to the diet the animals were rendered EFA and fat deficient. After 90 days on this diet the linoleic acid content of the epididymal fat pads determined by gas-liquid chromatography(4) fell to 0.5-2.0%, while in control pads 18-22% was found. At this time, *in vivo* incubation of epididymal fat pads, exteriorized with their vascular and neural connections intact, was carried out as described previously(2). The incubation medium (2 ml) consisted of Krebs-Ringer phosphate buffer (Ca^{++} omitted), pH 7.45, with 5 mM glucose, 0.7 mM dialyzed bovine serum albumin (Pentex, Inc.) and 0.7 mM labeled fatty acid. In mixtures of palmitic and linoleic acids equimolar concentrations were used. Incubation time was 20 minutes at 37°. Following incubation the pads were washed(2) and either both pads were removed or one

* Supported in part by research grant H-5705, Nat. Inst. Health, U.S.P.H.S.