Sarcomas After Inoculation of Newborn Hamsters with *Herpes virus hominis* Type 2 Strains[1] (34945)

ANDRÉ J. NAHMIAS,[2] ZUHER M. NAIB, WILLIAM E. JOSEY, FREDERICK A. MURPHY, AND CAROLYN F. LUCE

Departments of Pediatrics, Preventive Medicine, Pathology, and Gynecology and Obstetrics, Emory University School of Medicine and National Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333

It has recently been recognized that there are at least two antigenic types of *Herpes virus hominis* (HVH) with different biological properties and epidemiological features (1, 2). An association has also been noted between genital (type 2 HVH) infection and anaplastic lesions of the cervix by our group (3–5) and that of Rawls *et al.* (6). It was, therefore, of interest to ascertain the possible tumorigenicity of type 2 HVH in newborn hamsters.

Materials and Methods. Virus strains. Type 1 HVH. VR_3 (1) from brain (encephalitis); SHE from penis of 3-year-old boy; RAN from skin (eczema herpeticum), and TYL from cornea (keratitis).

Type 2 HVH. MS (1) from brain (multiple sclerosis); LOV, CUS, McD, EL, BEN, CUR, PIT, BER, DIC, COX, HUL, HOW and STO from male or female genitalia; HIC and ALA from skin (newborn). These strains, with the exception of VR_3 and MS, had been isolated in primary rabbit kidney tissue culture cells grown in Eagle's MEM and received 2–7 further passages in these cells prior to use.

Newborn hamsters. Pregnant Syrian hamsters from Lakeview Farms, Newfield, New Jersey, were housed individually. Newborn hamsters were inoculated with varying dilutions of virus (10^1 – 10^5 TCD_{50}) in 0.05 ml by one of several routes; subcutaneous (over the scapulae), intrathoracic (on the right side), or intraperitoneal. In addition, groups of newborn hamsters received ultraviolet-inactivated MS, BEN, or ALA viruses with initial titers of 10^5–10^6 TCD_{50}/0.05 ml; no infectious virus was demonstrable after ultraviolet treatment. Control animals included newborns receiving no inoculation or animals receiving extracts from noninfected primary rabbit kidney tissue cultures or Eagle's MEM. Initially, baby hamsters surviving over 3 weeks were separated according to sex. However, in one of the early experiments, a tumor was found 5 months after inoculation of a female hamster which had been sexed wrongly and had become pregnant at 3 months of age. For this reason, in later groups, about half the surviving females were allowed to become pregnant. Animals were observed at least once a week for the development of palpable masses, and those hamsters dying after the first 3 weeks were examined at autopsy for tumor formation.

Tumor transplantation and tissue culture preparation. Tumors were finely minced in Hanks' BSS and inoculated, via trochar, subcutaneously or intraperitoneally into newborn or weanling hamsters. Monolayer cell culture preparations from 3 original tumors or transplanted tumors were made by trypsinization; cultures were maintained in McCoy's medium (Grand Island) containing 10% calf serum.

Attempts to demonstrate virus by isolation in cell culture. Homogenates of primary tumors, transplanted tumors, and cell cultures derived from tumors were inoculated into primary rabbit kidney cell monolayers in tubes. These were examined for CPE over a period of 10 days.

[1] Supported by grants from the American Cancer Society and the Winship Clinic, Emory University.

[2] Recipient of Research Career Development Award IK-AI-18637 from the U.S. Public Health Service.

TABLE I. Tumors Observed After Inoculation of Newborn Hamsters with HVH Type 1 and Type 2 Strains.

	No. newborn hamsters	No. survived >3 weeks	No. still under observation	No. with known tumors/No. survived >3 weeks minus no. still under observation
Type 2 HVH strains				
Noninactivated virus (16)	1336	541	187	8/354 (2.3%)
Ultraviolet-inactivated virus (3)	245	178	147	1/31 (3.2%)
Type 1 HVH strains				
Noninactivated virus (4)	387	136	16	0/120 (0%)
Controls				
Eagle's MEM, cell material, no inoculation	267	173	72	1/101 (1%)
Total	2235	1028	422	10/606 (1.7%)

Attempts to demonstrate virus by electron microscopy. Primary and transplanted tumor tissues and cell cultures derived from them were fixed in 2.5% glutaraldehyde for 2 hr at 4°, treated with 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in Araldite–Epon. Sections were stained with uranyl acetate and lead citrate.

Antibody assays. Serum was obtained from hamsters with original or transplanted tumors and assayed for antibodies to type 1 (VR_3) and type 2 (MS) HVH by neutralization test.

Results. It was found early that virus doses over 10^3 TCD/50 of either type 1 or type 2 HVH produced 95–99% mortality. With lesser virus doses, which were then used for later experiments, no appreciable difference in mortality was found between type 1 and type 2 strains; about one-third of the inoculated animals survived over 3 weeks. Approximately three quarters of the hamsters receiving ultraviolet-inactivated viruses also survived 3 weeks after inoculation (Table I).

Since over one-third of animals are still under observation, the frequency of tumors observed is based on those animals surviving over 3 weeks after birth, minus the number still under observation. On this basis, the frequency of tumors observed (Table I) was 2.3% in animals inoculated with HVH type 2 strains and 3.2% in hamsters receiving ultraviolet-inactivated type 2 strains. None of 120 newborn hamsters inoculated with type 1 HVH strains developed tumors and 1 of 101 (1%) control animals developed a tumor. This animal had been inoculated 9 months previously with Eagle's MEM intrathoracically and was observed to have a cheek pouch tumor; on histological examination, this tumor was found to be a well-differentiated fibrosarcoma.

More detailed information on the tumors found after inoculation of HVH type 2 strains is summarized in Table II. Four tumors were associated with the MS type 2 virus, two with ALA, and one each with ELL and LOV. Dosages of noninactivated virus inoculated varied from $10^{1.5}$ to 10^3 TCD_{50}/0.05 ml. The tumor (No. 9) found in a hamster inoculated with ultraviolet-inactivated virus followed BEN type 2 strain inoculation. The tumors appeared at or close to the site of inoculation in all but one case; the one exception (No. 7) was an intraperitoneal tumor in an animal inoculated via the intrathoracic route.

The tumors appeared from 5 to 28 months after inoculation. Four occurred in male and five in female animals. The initial finding of earlier appearance of tumors in female hamsters allowed to become pregnant (Nos. 1 and 2) was not later corroborated (Nos. 7 and 8). The histological type of the nine tumors were all undifferentiated sarcomas, with two having tissue elements permitting them to be

TABLE II. Sarcomas After Inoculation of Newborn Hamsters with HVH Type 2 Strains.

Hamster		Virus			Tumor		
Ident. no.	Sex	HVH type 2 strain	Dose (TCD_{50}/0.05 ml)	Site of inoculation	Site	Time of appearance (months)	Histological type
1	F[a]	MS	$10^{3.0}$	Intrathoracic (rt.)	Ribs and subcut. (rt.)	5	Chondrosarcoma[b]
2	F[a]	MS	$10^{1.5}$	Intrathoracic (rt.)	Subcut. (rt.)	7	Malignant hemangioendothelioma
3	M	MS	$10^{3.0}$	Intrathoracic (rt.)	Intrathoracic (rt.)	14	Undifferentiated sarcoma
4	M	MS	$10^{1.5}$	Intraperitoneal	Intraperitoneal	28	Undifferentiated sarcoma
5	F	ALA	$10^{1.5}$	Intrathoracic (rt.)	Subcut. (rt.)	19	Hemangiopericytoma[c]
6	M	ALA	$10^{2.0}$	Intrathoracic (rt.)	Intrathoracic (rt.)	21	Osteochondrosarcoma
7	F[a]	ELL	$10^{2.0}$	Intrathoracic (rt.)	Intraperitoneal (rt.)	20	Undifferentiated sarcoma
8	F[a]	LOV	$10^{2.0}$	Intrathoracic (rt.)	Intrathoracic (rt.)	25	Undifferentiated sarcoma
9	M	BEN	$10^{5.0d}$	Intrathoracic (rt.)	Subcut. (rt.)	7½	Malignant hemangioendothelioma[c]

[a] Female hamsters allowed to become pregnant.
[b] Subcultivated in tissue culture—no C-type particles observed.
[c] Subcultivated in tissue culture—C-type particles observed.
[d] Before ultraviolet inactivation; no infectious virus after uv inactivation.

classified a chondrosarcoma or osteochondrosarcoma and three as hemangiopericytoma or malignant hemangioendothelioma.

Five of the tumors were found at autopsy; one was abscessed. Three tumors, however, were transplantable (Nos. 1, 5, and 9) and were subcultivated in tissue culture. From none of the tumors, transplants, or derived tissue culture cells were herpes viruses isolated. In none of three tumors examined by electron microscopy were herpes virus particles found. However, in two of these three tumors (Nos. 5 and 9) and their tissue cultures, C-type particles were demonstrated by electron microscopy (Fig. 1). Neutralizing antibodies to HVH type 1 or 2 could not be detected in serum obtained from animals with original or transplant tumors.

Discussion. Earlier attempts by Rapp and Falk (7) to produce tumors with HVH strains (typed by us as type 1) were unsuccessful. More recently, Trentin, *et al.* (10) were also unsuccessful in producing tumors in newborn hamsters with HF, a type 1 HVH strain (1). One of the problems found by these workers, and confirmed in our studies, is the high mortality rate obtained in newborn hamsters inoculated with relatively low virus doses.

It is not possible at present to be certain of the role of HVH type 2 in causing the sarcomas observed. No HVH virus was recoverable from the tumors and no HVH antibodies were demonstrable in the sera of animals with tumors. Attempts to demonstrate a relationship between these tumors and HVH by the use of complement-fixation, immunofluorescence, and cell-fusion techniques have so far yielded inconclusive results. The increased frequency of tumors in animals inoculated with type 2 virus strains relative to that in animals given type 1 HVH or in

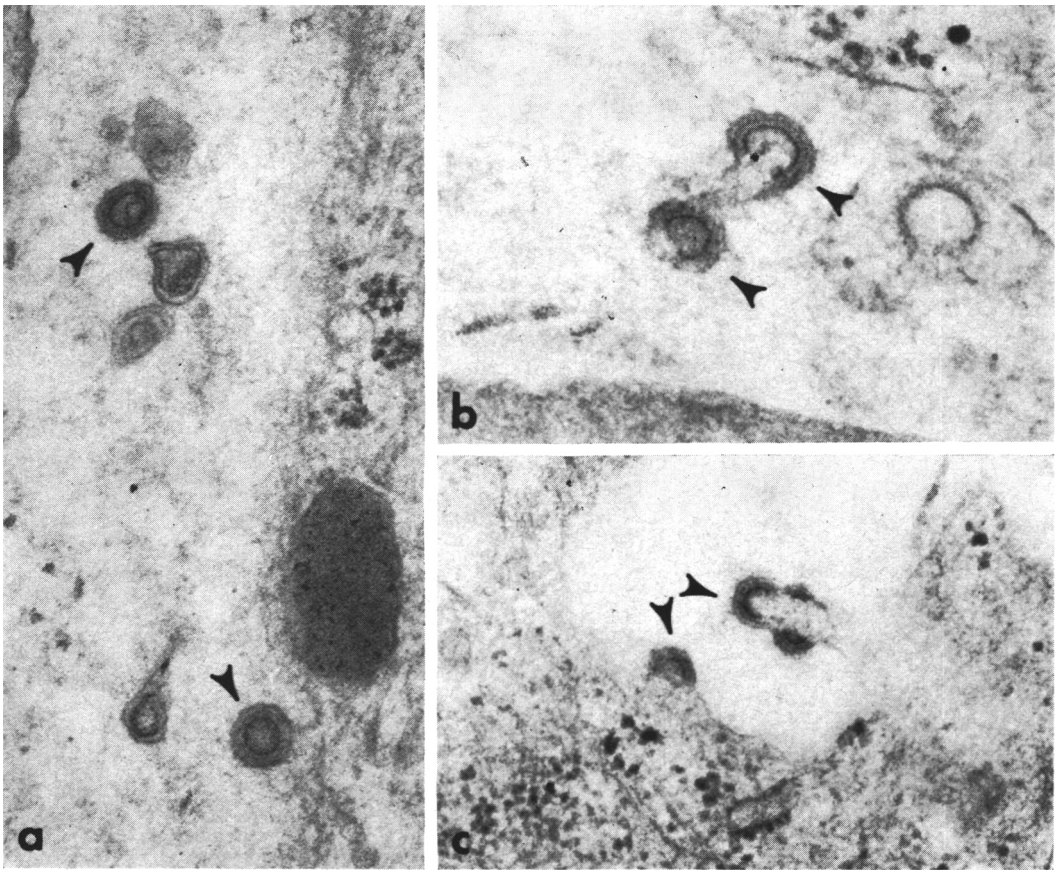

FIG. 1. a. C-type virus particles (arrows) in an extracellular space in hamster tumor No. 5. ×72,000. b. At higher magnification, particles (arrows) from an *in vivo* transplant of tumor No. 5 are resolved into typical C-type construction with triple shells. ×96,000. c. C-type virus particles (arrows) budding from the plasma membrane of a cell in tumor No. 9. ×68,000.

control animals, and the close relationship of the site of most tumors to the site of inoculation suggest some etiological association. The histological character of all the tumors associated with HVH type 2 inoculations was that of undifferentiated sarcoma; some of the tumors (*e.g.*, chondrosarcoma or hemangioendothelioma) are of unusual cell types as compared with tumors found in association with other viruses (8). The tumor found in the cheek pouch of one of the control animals was a low-grade well-differentiated fibrosarcoma.

Sera of tumor-bearing animals did not react with polyoma, SF_{40} or adenovirus types 12 or 18 antigens by complement fixation tests. However, the finding of C-type particles in two of three tumors examined by electron microscopy raises several questions related to their significance. Such C-type particles have been noted in a variety of spontaneous or viral-induced tumors (9). Work is in progress to determine whether the C-type particles observed in the hamster tumors are passenger viruses or if they are the causative agents of oncogenesis, possibly triggered after HVH type 2 inoculation.

Summary. Inoculation of newborn hamsters with virus doses greater than 10^3 TCD_{50} of types 1 or 2 *Herpes virus hominis* (HVH) strains caused a mortality approaching 100%. With lesser virus doses, nine sarcomas have been found 5–28 months after inoculation of four different

noninactivated HVH type 2 strains and one other ultraviolet-inactivated type 2 strain. None of the animals inoculated with HVH type 1 strains developed tumors, and one newborn hamster inoculated intrathoracically with Eagle's MEM developed a cheek-pouch well-differentiated fibrosarcoma. Eight of the nine tumors with HVH type 2 strains were at or close to the site of inoculation and the histological characteristics of some of these tumors differ from those associated with other viral-induced hamster sarcomas.

No clear-cut evidence of a relationship between HVH and the tumors by a variety of virological and serological assays has been obtained as yet. The finding of C-type particles in two of three tumors or their explants by electron microscopy has raised further questions about the role of HVH in the causation of the tumors found.

The authors are grateful to Mrs. Alyne K. Harrison for assistance in electron microscopy and to Dr. R. Gilden for performing the serological tests with SV_{40}, polyoma, and adenoviruses.

1. Dowdle, W. R., Nahmias, A. J., Harwell, R. W., and Pauls, F. P., J. Immunol. **99**, 974 (1967).

2. Nahmias, A. J., and Dowdle, W. R., Progr. Med. Virol. **10**, 110 (1968).

3. Naib, Z. M., Nahmias, A. J., and Josey, W. E., Cancer **19**, 1026 (1966).

4. Naib, Z. M., Nahmias, A. J., Josey, W. E., and Kramer, J. H., Cancer **23**, 940 (1969).

5. Nahmias, A., Naib, Z., and Josey, W., Perspect. Virol. **7**, 1970 (in press).

6. Rawls, W. E., Tompkins, W. A., and Melnick, J. L., Amer. J. Epidemiol. **89**, 547 (1969).

7. Rapp, F., and Falk, L. A., Proc. Soc. Exp. Biol. Med. **116**, 361 (1964).

8. Berman, L., J. Nat. Cancer Inst. **39**, 847 (1967).

9. Stenback, W. A., Van Hoosier, G. L., and Trentin, J. J., Proc. Soc. Exp. Biol. Med. **122**, 1219 (1966).

10. Trentin, J. J., Van Hosier, G. L., Ferguson, D. G., and Kitamura, I., Proc. Soc. Exp. Biol. Med. **132**, 912 (1969).

Received Mar. 2, 1970. P.S.E.B.M., 1970, Vol. 134.