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ANALYSES OF TRANSPLANTED MURINE TUMORS FOR HSV DNA SEQUENCES

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Meignier *et al.* (1986) report the results of exposure of C57BL/6NCr mice to vaginal plugs containing live or inactivated herpes simplex virus 1 or 2 (HSV-1 or HSV-2) or recombinant viruses 5 times a week for up to 114 weeks. Genital organs showing abnormalities were transplanted into nude mice. Of 33 transplants, 13 produced subcutaneous tumors in nude mice and 12 were subsequently transplanted into C57BL/6NCr mice. We report that the DNA extracted from coded tumor tissues of nude mice and from normal viscera of the same rodents did not hybridize with HSV-1 and HSV-2 DNA probes representing the viral genomic regions shown previously to be capable of morphologically transforming cells in culture. The sensitivity of the assays was such that we could detect 0.5 copies of the HSV sequences of complexity equal to or greater than 1 Kbp per cell DNA equivalent. To control for the sensitivity of the assays in the actual hybridizations, the tumor-cell DNA was also hybridized with a β-globin mouse DNA probe. A striking feature of these control hybridizations was the detection of β-globin polymorphism in some nude mouse tumors. The β-globin polymorphism allowed us to conclude that the analyzed tissues contained significant amounts of the tumor cells occurring in the C57BL/6NCr mice.

In the companion report, Meignier *et al.* (1986) show the results of studies involving prolonged exposure of C57BL/6NCr mice to live or inactivated wild-type and recombinant herpes simplex viruses (HSV) in cotton plugs inserted into the vagina 5 times a week for up to 114 weeks. That investigation failed to reveal a causal relationship between the induction of genital tumors confirmed by histopathology and exposure to HSV. It was of interest, nevertheless, to determine whether the tumors appearing in genital tissues of mice exposed to live or inactivated virus retained HSV DNA sequences. Since the tumors were small, it seemed desirable both to verify their malignant characteristics and to augment the amount of cancerous tissue available for study by transplantation into nude mice. We now describe the results of these studies.

MATERIAL AND METHODS

Nude mice

HO-CD-1 Swiss outbred mice or HO BALB/c nude mice were obtained from Charles River (Kingston division, Stoneridge, New York) and NCR-Nu mice were obtained from the National Cancer Institute (Frederick Cancer Facility). The mice were used at an age of 8 to 22 weeks.

Transplantation of C57BL/6NCr tumor fragments into nude mice

Genital tumors detected at autopsy and measuring at least 5 mm in diameter were excised aseptically so that approximately two-thirds of the tumor was removed, leaving the remaining tumor tissue attached to the genital tract for histopathologic examination. The excised tissue was transferred to a Petri dish containing cell culture medium 199V consisting of mixture 199 supplemented with 1% inactivated calf serum. After the connective tissue had been trimmed away, the excised portion of the tumor was cut into small fragments approximately 1 mm^3 in volume, and implanted subcutaneously (s.c.) into the right axillary region of 2–5 nude mice.

Spontaneous death rates in the mice receiving transplants (15% during the entire project) were similar to those in control mice (12%). Animal weight and tumor size were followed weekly. Animals in which no tumor development was observed or in which an observed nodule did not increase in size were killed after 15 to 20 weeks. This interval was chosen on the basis of observation of tumor development in the first 10 sets of transplants.

Tumor-bearing animals were killed when the tumor reached a diameter of at least 10 mm. During autopsy, viscera (liver, spleen, kidneys) and any detected spontaneous or possibly metastatic tumors were taken for histopathology and storage at −70°C, then the entire primary tumor was removed aseptically into a Petri dish containing medium 199V. After trimming, the tissue was divided into portions for serial transplantation studies and analysis for HSV DNA sequences. The latter portions were stored at −70°C until shipment to the University of Chicago.

The second serial passage was done in female C57BL/6NCr mice which were at least 8 weeks old. One mouse from each set of successful transplants was killed when the tumor reached at least 10 mm in diameter. Tumor and viscera were removed and processed as above, but were not analyzed in these studies.

Preparation of mouse tissue DNA

Frozen specimens were ground with mortar and pestle under liquid nitrogen. The fine tissue fragments were then incubated overnight at 37°C, with gentle shaking in the presence of 15–30 ml of lysis buffer [0.1M NaCl, 0.01M Tris, pH 8.0, 0.01M EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.2 mg proteinase K per ml]. The lysates were then extracted several times with phenol, chloroform-2% isoamyl alcohol, and several times with chloroform-2% isoamyl alco-

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TABLE I - HISTORY AND TRANSPLANTABILITY OF THE C57BL/6NCr TUMORS USED FOR TRANSPLANTATION AND OF RESULTANT NUDE MOUSE TUMORS THAT WERE TESTED FOR THE PRESENCE OF HSV DNA

		C57BL/6NCr mouse history				History of nude mouse		
Mouse group[1]	Treatment[2]	C57BL-6NCr mouse number	Weeks of treatment prior to excision	Transplanted tissue	Mice + tumors/mice transplanted	Nude mouse number	Week when first palpable, (week when tumor excised)	Transplantability in C57BL/6NCr mice
A	No pellets	470	108	Uterine tumor	3/3	470-3	5 (12)	4/4
A	No pellets	471	108	Ovarian tumor	0/2			
B	Non-infected cells	103	75	Cervical tumor	3/3	103-1	7 (11)	NT[4]
B	Non-infected cells	179	87	Gen. abnorm.[3]	0/1			
C	Non-infected cells	189	87	Uterine tumor	5/5	189A-3	4 (27)	2/2
C	Non-infected cells	438	104	Ovarian tumor	0/5			
E	G-UV	261	88	Uterine tumor	0/5			
E	G-UV	493	107	Ovarian tumor	0/5			
H	F-UV	268	88	Ovarian tumor	3/3	268-1	3 (10)	3/3
H	F-UV	629	105	Cervical tumor	0/4			
I	G-UV	96	64	Uterine tumor	5/5	96-1	8 (10)	3/3
I	G-UV	566	101	Cervical tumor	2/5	566-4	8 (18)	4/5
J	F-live	864	94	Ovarian tumor	0/2			
J	F-live	579	87	Uterine tumor	1/5			
J	F-live	768	92	Gen. abnorm.[3]	0/4			

(continued)

TABLE I (continued) – HISTORY AND TRANSPLANTABILITY OF THE C57BL/6NCr TUMORS USED FOR TRANSPLANTATION AND OF RESULTANT NUDE MOUSE TUMORS THAT WERE TESTED FOR THE PRESENCE OF HSV DNA

C57BL/6NCr mouse history						History of nude mouse		
Mouse group[1]	Treatment[2]	C57BL-6NCr mouse number	Weeks of treatment prior to excision	Transplanted tissue	Mice + tumors/mice transplanted	Nude mouse number	Week when first palpable, (week when tumor excised)	Transplant-ability in C57BL/6NCr mice
K	A5C-live	757	110	Uterine tumor	0/5			
L	A5C-UV	279	89	Uterine tumor	4/4	279-1	7 (18)	3/4
						279-3	7 (21)	NT[4]
L	A5C-UV	565	102	Uterine tumor	5/5	565-1	5 (12)	4/4
L	A5C-UV	625	105	Cerv./vag. tumor	0/1			
M	D4E1-live	137	77	Gen. abnorm.[3]	0/1			
M	D4E1-live	454	101	Uterine tumor	2/5	454-1	12 (17)	3/3
N	D4E1-UV	123	67	Uterine tumor	5/5	123-1	3 (5)	4/4
N	D4E1-UV	849	107	Uterine tumor	0/1			
O	D5E1-live	380	91	Ovarian tumor	5/5	380-1	7 (15)	4/4
O	D5E1-live	830	109	Ovarian tumor	0/2			
Q	D4E1-live G UV	368	95	Uterine tumor	0/4			
Q	D4E1-live/G UV	772	111	Uterine tumor	0/3			
R	D4E1-UV/G UV	687	103	Ovarian tumor	0/4			
R	D4E1-UV/G UV	734	104	Uterine tumor	5/5	734-2	4 (17)	3/4
R	D4E1-UV/G UV	828	106	Ovarian tumor	0/6			
R	D4E1-UV/G UV	867	108	Gen. abnorm.[3]	0/2			
R	D4E1-UV/G UV	876	108	Gen. abnorm.[3]	0/2			
R	D4E1-UV/G UV	878	108	Gen. abnorm.[3]	0/3			

[1]Designations of the mouse groups are the same as in Meignier *et al.* (1986).–[2]The procedures for virus preparation and for mouse treatment are described in Meignier *et al.* (1986).
[3]Genital abnormality such as thickening of vaginal walls or cystic dilatation of uterine horns.–[4]Not tested.

TABLE II - TRANSPLANTABILITY IN NUDE AND SUBSEQUENTLY IN C57BL/6NCr MICE OF VARIOUS TISSUES EXCISED FROM C57BL/6NCr MICE[1]

Transplanted tissue	Total specimens tested	Number of tumors with positive transplants in nude mice	Number of tumors with positive transplants in C57BL/6Nr mice
Cervical tumors	4	1	1[2]
Uterine tumors	14	9	9
Ovarian tumors	9	2	2
Genital abnormalities	6	0	0

[1]Summary of results presented in Table I.-[2]Only one tumor transplanted back from nude to C57BL/6Nr mice.

hol. The aqueous phase was then mixed with 0.1 volume of 3M sodium acetate, and 2 volumes of ethanol. High-molecular-weight DNA was spooled on a glass rod.

Preparation of DNA for reconstruction tests

HSV-1 (Justin) and HSV-2 (333) DNAs for use in the reconstruction experiments were purified from infected HEp-2 cells by CsCl equilibrium density centrifugation as previously described (Locker and Frenkel, 1979). Mouse Ltk$^-$ cells grown in 32-oz bottles were rinsed with buffered saline (0.15M NaCl, 0.02M Tris-HCl pH 7.8) treated with lysis buffer, and processed for extraction of high-molecular-weight DNA as described above. Reconstruction mixtures equivalent to 10, 1 and 0.5 copies per cell consisted of 10 μg of Ltk$^-$ cell DNA mixed with 2×10^{-3}, 2×10^{-4}, and 10^{-4} μg of HSV DNA, respectively.

Hybridizations

Approximately 10 to 15 μg of tissue DNA were digested with 20 units of restriction enzyme (BRL, Gaithersburg, MD) per μg of DNA at 37°C for 6 hr according to manufacturer's specifications. One lane in each gel contained phage lambda DNA digested with HindIII to serve as a size marker. The reconstruction and test tumor DNAs probed with labelled BamHI V and BamHI G were digested with BamHI, DNAs probed with BglII O were digested with HindIII + BamHI, DNAs probed with BglII N were digested with BamHI, and DNAs probed with BglII C were digested with BamHi or HindIII + BamHI. After electrophoresis in 0.6% agarose, the gels were stained with ethidium bromide, treated with alkaline and neutralizing solutions and then transferred to a nitrocellulose sheet (Schleicher and Schull) as described by Southern (1975), except that the transfer was done in the presence of 20× SSC (1× SSC contains 0.15M NaCl, 15 mM sodium citrate). After the transfer the nitrocellulose sheets were rinsed with 2× SSC and baked at 80°C for 5 hr. Prior to hybridization, the nitrocellulose sheets were incubated (0.3 ml/cm^2) at 65°C for 6 hr in hybridization buffer containing 6× SSC, 2× Denhardt's solution, 30% formamide, 10% dextran sulfate, 0.5% SDS, and 200 μg of sonicated, denatured salmon sperm DNA per ml. Hybridization was done for 36 hr with 2×10^6 cpm of ^{32}P-labelled probe per ml of hybridization buffer (0.1 ml/cm^2). Hybridization temperature was 65°C for the HSV-cloned DNA probes and 45°C for the mouse β-globin probe. Following hybridization, the nitrocellulose sheets were rinsed at room temperature with rinsing solution consisting of 6× SSC, 1× Denhardt's solution, 30% formamide, and 0.5% SDS. Removal of non-hybridized probe was done by incubation with the same solution at the hybridization temperature (4 hr), 3 changes of 2× SSC, 0.1% SDS (1.5 hr each) and two changes of 0.1× SSC, 0.1% SDS (1 hr each). The sheets were air dried and autoradiograms were made on Kodak X-Omat XS-5 film at −70°C with intensifying screens.

Preparation of probes

The plasmids p-24, p-29 and pNF171 were derived by cloning the corresponding BglII fragments of HSV-2(333) DNA into the BglII site of pKC7 (Rao and Rogers, 1979). P-24 contained the BglII C fragment, p-29 contained the BglII N fragment, and pNF171 the BglII O fragment (Spaete and Frenkel, 1985). The plasmid pNF242 contained the BamHI V fragment of HSV-1 (Patton) DNA cloned into the BamHI site of pUC9. The plasmid pRB102 contained the BamHI G fragment of HSV-1(F) DNA (Post *et al.*, 1980). The plasmid pCR1-βM9 (Rougeon and Mach, 1977) was a generous gift from Dr. S. Weaver, University of Illinois. This plasmid contained a cDNA mouse β-globin insert.

The probes were labelled by nick translation in the presence of ^{32}P-labelled dGTP and dCTP (Amersham, 800 Ci/mM), to a specific activity of 2×10^8 cmp/μg of DNA. Probes were denatured at 110°C for 10 min in TE buffer (0.01M Tris, pH 7.8, 0.001M EDTA).

RESULTS

Studies of genital tumors

On the basis of expectation that the cervical tumors developing in the C57BL/6NCr would be large, the initial experimental design involved subdivision of each tumor tissue into 3 portions to be used for: (i) histopathological studies, (ii) DNA hybridizations, and (iii) transplantation studies in nude mice. However, most of the tumors detected by gross examination at the time of autopsy were small. Therefore, where feasible, fragments of the tissue suspected to contain malignant cells were transplanted into nude mice and resultant tumor tissues were used for the hybridization studies.

A total of 33 specimens were selected for transplantation into nude mice solely on the basis of size and abnormal appearance and irrespective of the genital site from which they were excised. Thirteen of these 33 specimens yielded tumors following transplantation into nude mice (Tables I and II). Following excision of these tumors, a portion of each tumor tissue was frozen for hybridization studies and another portion was transplanted into C57BL/6NCr mice. All of these sec-

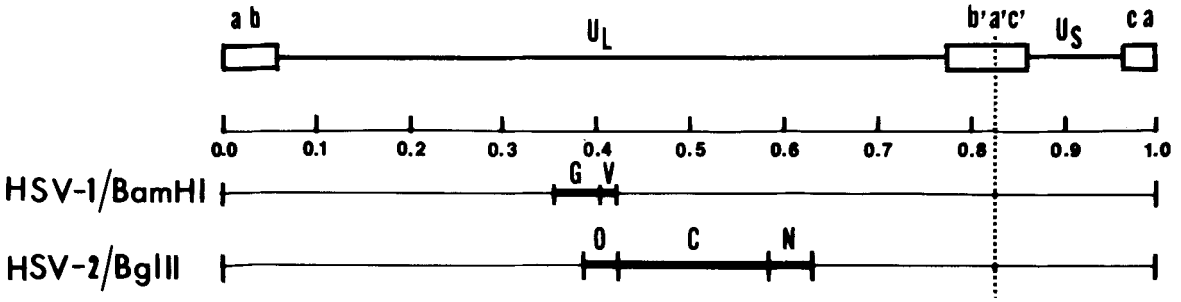

FIGURE 1 – Diagrammatic representation of map locations of HSV DNA fragments used as hybridization probes.

ond-generation transplants grew in C57BL/6NCr mice (Tables I and II). One hypertrophic lymph node found in one of the mice (566-4) was also excised and included in the analyses. Visceral tissues were excised from the tumor-bearing nude mice to serve as controls in the hybridization studies. The source and history of the 13 transplantable tumors obtained in nude mice are detailed in Table I.

Selection of HSV, DNA probes

Because the hybridization studies were done under code, all DNA samples were tested with both HSV-1 and HSV-2 DNA probes. For this reason, the probes were selected to represent domains of HSV-1 and HSV-2 genomes shown previously to morphologically transform cells in culture. Figure 1 summarizes the map locations of the HSV-1 and HSV-2 cloned DNA fragments selected as probes. Specifically, an equimolar mixture of BamHI V and BamHI G was selected to represent the HSV-1 morphological transforming region 1 (mtr1; Camacho and Spear, 1978; Reyes *et al.*, 1980); BglII O was selected to represent the corresponding sequences of the HSV-2 DNA. BglII N (Reyes *et al.*, 1980; Galloway and McDougall, 1981; Galloway *et al.*, 1984) and BglII C (Jariwalla *et al.*, 1980) represented the two morphological transforming regions (mtr2 and mtr3, respectively) of HSV-2 DNA.

Hybridization design and detection levels

Because viral DNA may be integrated in non-unique locations in cellular DNA, we chose to digest the test DNAs with enzymes known to generate one or more small fragments from the region represented by each of the probes.

Two types of control hybridizations were done in order to ensure an adequate level of sensitivity. First, mixtures of Ltk$^-$ DNA and HSV-1 or HSV-2 DNAs were prepared to reconstruct ratios of 10, 1 and 0.5 copies per cell. For each probe the DNA in the reconstruction mixtures and the test DNAs were digested with the same restriction enzyme. Second, DNA preparations from each of the mouse tissues were probed with a β-globin probe so as to verify our ability to detect single-copy sequences in each of the DNA preparations. This test also ascertained that there was no extensive degradation of the DNA extracted from the mouse tissues.

Representative results of these tests, shown in Figures 2–4, revealed the following: (i) the sensitivity of the hybridization assays exceeded 0.5 copies per cell, inasmuch as hybridization bands could be seen with each of the probes in the 0.5 copies per cell reconstruction mixtures (Fig. 2); (ii) the HSV-1 DNA probes hybridized more efficiently to reconstruction mixtures containing HSV-1 DNA, whereas the HSV-2 DNA probes hybridized more efficiently to the HSV-2 reconstruction mixtures; (iii) in all cases the β-globin probe hybridized to several bands in the mouse DNA indicating that the level of sensitivity permitted the detection of a single-copy DNA sequences (Fig. 4); (iv) hybridization of the β-globin probe with the various mouse tissue DNAs yielded variable patterns reflecting β-globin polymorphism. As described below, this polymorphism permitted (in some instances) the

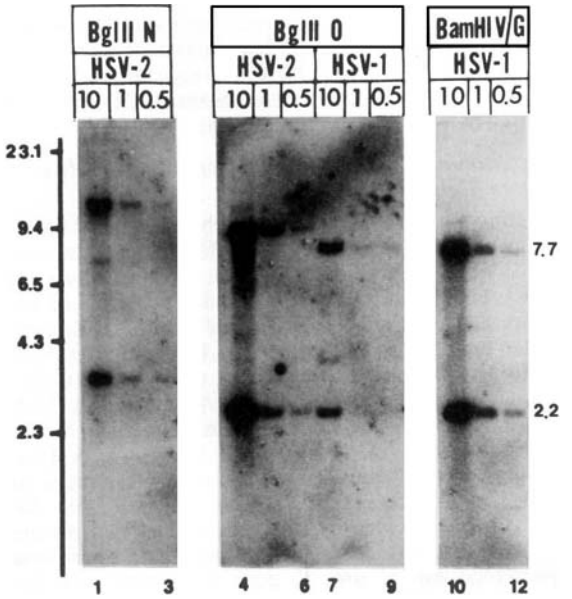

FIGURE 2 – Autoradiographic images showing reconstruction experiments involving hybridizations of the BglII N, BglII O, and BamHi V+G probes to nitrocellulose sheets containing 10 μg per lane of mixtures of Ltk$^-$ DNA and HSV-1 or HSV-2 DNAs at ratio equivalents to 10, 1 and 0.5 copies per cell. Numbers to the left and right of the figure represent DNA sizes in kb.

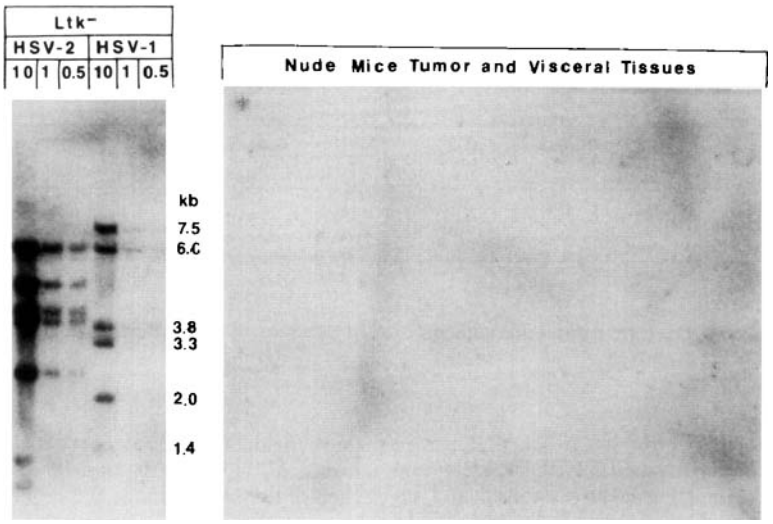

FIGURE 3 – Autoradiographic images of hybridizations with the HSV-2(333) BglII C DNA probe. Left: Reconstruction tests employing ^{32}P-labelled p-24 DNA probe in hybridizations to BamHi-digested reconstruction mixture DNAs prepared as described in "Material and Methods." Right: Hybridization of the p-24 (BglII-C)-labelled probe to DNAs extracted from visceral and tumor tissues of the transplanted nude mice.

identification of the origin of cells contained in the excised tumor specimens.

Hybridization of the tumor and visceral tissue DNAs with HSV-1 and HSV-2 probes

None of the hybridizations of HSV-1 and HSV-2 DNA probes with DNAs from tumors and visceral tissues excised from nude mice yielded visible bands, notwithstanding lengthy autoradiographic exposures. An example of one set of hybridizations done with ^{32}P-labelled HSV-2(333) BglII-C fragment is shown in Figure 3. Similar results were obtained with all other HSV DNA probes (data not shown).

Hybridization of the tumor and visceral tissue DNAs with the β-globin probe

Figure 4 shows the pattern of hybridization of the β-globin probe to BamHI-digested DNA from two untreated nude mice (lanes 1 and 2), from an untreated C57BL/6NCr mouse (lane 3), and from each of the tumors and corresponding visceral tissues of the implanted nude mice (lanes 4 to 28). The results of these hybridizations can be summarized as follows. (i) Hybridization to the untreated C57BL/6NCr mouse DNA yielded 4 bands estimated to contain fragments 11, 7.2, 5.6, and 2 kb in size (Fig. 4, lane 3). This hybridization pattern has been designated as the A pattern in Table III. The observed sizes agreed well with the BamHI map provided by Weaver *et al.* (1981) for the β-globin region of C57BL/10 mouse. The 4 bands thus represented the 5′ and 3′ portions (defined by the BamHI site) of the two adult globin genes inasmuch as BamHI cleaves once within the coding region of the β-globin gene. As expected, no hybridization was observed with the other members of the globin gene family, due to the stringent hybridization conditions employed. (ii) Hybridization of the β-globin probe to the DNA of one of the untreated nude mice (Fig. 4, lane 1) yielded 3 bands of approximate sizes 7.2, 4.3 and 1.1 kb. These bands were collectively designated as the B pattern in Table III. To our surprise, the hybridization with DNA from the second untreated nude mouse obtained from the same animal supplier yielded a mixture of the A and B patterns (Fig. 4, lane 2). The pattern obtained with this mouse was therefore designated as the AB pattern. All of these bands represented the products of limit digests of host DNA with the restriction endonucleases. This conclusion is based on the results of control experiments involving deliberate partial digest tests (data not shown). (iii) the hybridization patterns of the β-globin probe to the BamHI-digested DNAs prepared from the viscera of the tumor-bearing nude mice fell into 3 groups. Thus, two mice (lanes 5 and 28) yielded the A pattern, two (lanes 16 and 22) yielded the B pattern, and the remaining 6 visceral DNAs (lanes 7,10,14,18,20, and 25) yielded the AB patterns. The simplest interpretation of these results was that the nude mice were not genotypically homogeneous inasmuch as the β-globin loci in these mice represented homozygous A, homozygous B, and heterozygous AB patterns. (iv) Irrespective of the β-globin DNA pattern of the visceral DNA, all β-globin patterns of the corresponding tumor DNAs were either of the A type or exhibited a mixed pattern in which the A bands predominated (Fig. 4 and Table III). Hybridization of DNA extracted from the hypertrophic lymph node excised from mouse 566-4 yielded the β-globin A pattern.

DISCUSSION

Association of HSV DNA with murine genital cancers

In the accompanying report, Meignier *et al.* (1986) described the development of tumors in C57BL/6NCr mice exposed to live and inactivated wild-type recombinant HSV viruses, as well as in mice exposed to lysates of uninfected cells. Although a relationship

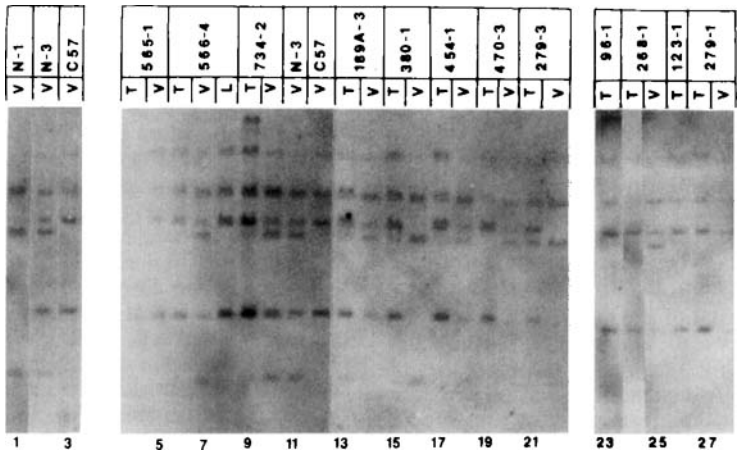

FIGURE 4 – Autoradiographic images of hybridizations with the pCR1-M9 probe carrying the mouse β-globin cDNA sequences (Rougeon and Mach, 1977) to nitrocellulose sheets containing 10–15 μg of BamHI-digested DNA per lane. V and T denote visceral and tumor tissues prepared from nude mice. L designates hypertrophic lymph node. Nude mouse designations are as in Table III. N-1 and N-3 represent untreated nude mice. C57 denotes a C57BL/6NCr mouse which received no vaginal cotton plugs.

between exposure to HSV and histopathologically proven cancers could not be demonstrated, it was of interest to establish whether genital tumors in animals exposed to live or inactivated HSV contained HSV DNA sequences. The original reports by Wentz *et al.* (1975; 1981*a*,*b*, 1983), did not include analyses of murine tumors for the presence of HSV DNA sequences.

In order to obtain sufficient amounts of tumor tissues for these analyses, we transplanted genital tissues from 33 tumors, obtained in C57BL/6NCr mice, into nude mice. Tissues were selected for transplantation on the basis of abnormal enlargements of the genital organs. At the time of transplantation these tissues had not been examined histologically and were therefore not confirmed to contain neoplastic growths. The 13 tumors successfully transplanted in nude mice were analysed for HSV DNA sequences. Reconstruction experiments employing a battery of selected probes have shown that the level of sensitivity attained in the hybridization tests was sufficient to detect viral DNA fragments in the size range of 1 to more than 10 kb, present at 0.5 copies/cell. Furthermore, hybridizations of each of the DNA preparations with a ^{32}P-labelled β-globin probe revealed that the extracted DNAs were reasonably intact and that single-copy DNA sequences could be detected by autoradiographic exposures of several days (*e.g.* 4 days for the results shown in Fig. 4).

Notwithstanding these precautions, we did not detect hybridization of mouse tumor DNA to the probes representing HSV-1 and HSV-2 DNA sequences reported previously to morphologically transform cells in culture.

Central to the results presented in this report are the following findings. (i) The evidence for a causal link

TABLE III – RESULTS OF THE β-GLOBIN HYBRIDIZATION TESTS

C57BL/6NCr mouse group[1]	Nude mouse number	β-globin DNA patterns in the hybridization tests[2]		
		S.c. tumor[4]	Viscera	Lymph node[3]
A	470-3	A	A B	
C	189A-3	A (B)	A B	
H	268-1	A	A B	
I	96-1	A	NT[5]	
I	566-4	A	A B	A
L	279-1	A	A	
L	279-3	A B	B	
L	565-1	A	A	
M	454-1	A	A B	
N	123-1	A	NT	
O	380-1	A (B*)	B	
R	734-2	A (B*)	A B	
Nude control	1		B	
Nude control	3		A B	
C57 control			A	

[1]Groups are designated as in Table I.–[2]Patterns A and B represent the hybridization band patterns as defined in the text. A B designates approximately equal molar ratios of the A and B patterns. A (B) represents higher intensities of the A band patterns, A (B*) represents mostly A pattern and only very faint B bands.–[3]Hypertrophic lymph node suspected to contain metastasis on the basis of external appearance at autopsy.–[4]S.c., subcutaneous.–[5]NT, not tested.

between HSV infection and cervical cancer must rest on a proven epidemiologic relationship. The finding of HSV DNA sequences, *per se*, is not proof of a causal relationship inasmuch as the progenitor of the cancer cells could have been infected with virus incapable of expressing lytic functions. In the absence of a relationship between development of genital cancers in mice demonstrated in the studies by Meignier *et al.* (1986) and of HSV DNA sequences in cancer cells, the issue concerning the fate of HSV DNA introduced into the genital organs in the studies described by Wentz *et al.* (1975) and Meignier *et al.* (1986) remains moot.

(ii) The interpretation of the negative results is not, however, straightforward. There are at least 3 alternative explanations for our failure to document the presence of HSV DNA sequences associated with morphological transformation in the mouse tumor cells. First, the sequence of HSV DNA retained in the cancer cells could be too small to be detected by our assays. Thus, if the maintenance of the transformed state required a relatively small segment such as, for example, a viral gene promoter significantly less than 1 kb, it would not be detected. Second, as proposed by Galloway and McDougall (1983), HSV-mediated transformation might involve the transient presence and subsequent loss of viral DNA from the transformed cells. If this were the case, the apparent absence of HSV DNA sequences in transplantable tumors would imply the existence of a strong selective pressure favoring the emergence of cells retaining a lower genetic complexity of HSV DNA sequences. Consistent with this model, earlier studies have demonstrated that the complexity of HSV DNA sequences decreased during propagation of transformed cells in culture or *in vivo* (Minson *et al.*, 1976; Frenkel *et al.*, 1976). Despite reports of an apparent decrease in complexity, HSV DNA sequences have been invariably detected in the transformed cells and tumors. The third possible explanation for the absence of detectable HSV DNA sequences in the mouse tumors is that viral DNA sequences were not involved in the events leading to generation of tumors in the treated mice. The absence of a relationship between histopathologically confirmed tumors and exposure to HSV (Meignier *et al.*, 1986) is consistent with this conclusion.

(iii) HSV DNA sequences have been sporadically reported in cervical cancers (Frenkel *et al.*, 1972; Park *et al.*, 1983; Galloway and McDougall, 1983; Prakash *et al.*, 1985). In a recent study employing the same probes and hybridization procedures similar to those described here, 2 of 10 tested human genital tumors, and 1 of 5 genital early dysplasias were found to contain subsets of the HSV-2 BglII O and BglI N sequences. Sequences homologous to the adjacent fragments were not detected (Manservigi *et al.*, 1986). Thus, at least in some situations involving the human host, the presence of selected subsets of viral DNA sequences in tumor cells was compatible with cell survival. In the absence of epidemiological evidence supporting a causal relationship between HSV and human malignancy, such sporadic findings of viral DNA sequences in a fraction of genital malignancies does not differentiate between the hypothesis that cancer cells are capable of maintaining HSV DNA sequences and the recent hypothesis by zur Hausen (1982, 1983) that HSV might serve as one of the as yet unidentified co-factors in the generation of papillomavirus-induced cervical malignancies.

Host markers in transplanted cancer cells

A striking feature of our results is the finding that the nude mice employed in this work were not homozygous with respect to β-globin sequences. In fact, the distribution of globin genotypes in tumor versus visceral tissues of the same mice provided the means by which we could estimate the proportion of cells representing progeny growth of the implanted tumor tissue. Thus, in 8 instances, the tumor tissues excised from nude mice with a homozygous B pattern or heterozygous AB pattern of β-globin showed predominantly the A pattern characteristic of the C57BL/6NCr mice, indicating that a majority of cells in the tumor tissue arose from the input implanted cells. Moreover, in one case (566-4) a hypertrophic lymph node proved to consist of metastatic C57BL/6NCr cells.

In this study β-globin polymorphism was found to be a very powerful experimental tool for identification of the origin of cells in both the primary implants and metastases in animals implanted with genetically dissimilar tumor tissue.

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