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Neoplastic Transformation of the Rat Visceral Yolk Sac by Polyoma Virus

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Abstract—*The transforming activity of polyoma virus was verified* in vitro *in organ cultures of 12-day old rat visceral yolk sacs and embryos. Organ cultures of 18-day old fetal organs were also included. The transforming capacity of the virus was found to be restricted to the endothelial cells of the rat visceral yolk sac. The neoplastic endothelial cells are readily transplantable and possess the polyoma tumor-specific transplantation antigens (TSTA).*

INTRODUCTION

WE RECENTLY described the development of hemangiomas in ectopic grafts of rat visceral yolk sacs and embryos after *in vitro* infection with polyoma virus [1]. No other polyoma-induced tumors were recorded. A similar observation was made in fetectomized rats injected with this virus into the placentas [2]. Also, in these experiments the tumors observed were all of vascular origin. This might indicate that the polyoma virus displays a particular tropism for the endothelial cells in the rat. One cannot, however, exclude the influence of the host in these *in vivo* experiments. Indeed, in young or adult immunosuppressed rats injected with polyoma virus the only tumors observed are hemangiomas, while rats inoculated at birth will mainly develop lipo-, osteo- and fibrosarcomas [3, 4]. To verify which tissue is preferentially transformed, independently of host reaction, an *in vitro* model is therefore necessary. We have shown that the rat visceral yolk sac can be kept in good conditions in organ culture for at least 4 weeks. During this period both the endodermal and mesodermal cells proliferate and thereafter differentiate into various adult tissues [5]. This model seemed to us suitable (i) to test the sensitivity of the proliferating cells for polyoma virus transformation and (ii) to verify the influence of this virus on the differentiation capacity of this extra-embryonic fetal membrane. In the present experiment we show that the endothelial cells of the rat yolk sac maintained in organ culture are indeed particularly sensitive to the transforming capacity of the polyoma virus and that the virus does not interfere with the differentiation capacity of this membrane into various well differentiated tissues.

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MATERIALS AND METHODS

Virus

Polyoma virus (Toronto strain, small plaque) was grown in mouse embryo fibroblast (MEF) cultures and prepared by the method of Crawford [6]. The virus was titrated by the plaque method of Dulbecco and Freeman [7] on mouse fibroblast cultures. The virus pool had a titer of 2×10^9 PFU/ml in Eagle's minimum essential medium (MEM). One-millilitre aliquots were stored in sealed glass ampules at −90° C. The control medium was prepared from MEF cultures not infected with polyoma virus.

Organ and tissue culture

Rats of the inbred R/A (Wistar albino) were used. Twelve days after mating (the day when copulation plug was found was counted as day 0) the uteri with embryos were removed and put in Petri dishes with PBS. The visceral yolk sac was dissected free from the Reichert membrane, amnion and placenta as previously described [5]. Pieces of visceral yolk sac and embryo were incubated for 2 hr in 1 ml of polyoma virus or control medium and rinsed carefully. After this treatment the tissues were incubated for 48 hr in a roller system and then put in organ culture in NCTC medium containing 20% horse serum [5]. Pieces of fetal gut, skin, cartilage and kidney obtained from 18-day old R/A fetuses were also included. They were treated in the same way as the 12-day old visceral yolk sac and embryo organ cultures. After 3 weeks in organ culture part of the pieces were fixed in formol for histological examination, part of them were put into culture dishes (Falcon 3803) to prepare monolayer cell cultures and part of the organ cultures were also grafted under the kidney

capsule of 6-week-old female R/A rats. The latter animals were killed 4 weeks after grafting. The cells which grew out as monolayers from the organ cultures put in culture dishes were injected subcutaneously into syngeneic 3-week old female R/A rats. The tumors which developed at the site of inoculation were fixed in formol and embedded in paraplast. The sections were stained with H and E, PAS and Tibor-Pap for reticulin.

Immunofluorescence

For the detection of the Factor VIII-related antigen cells growing on coverslips or harvested after trypsinization and centrifuged in a cytocentrifugator (Shandon Scientific Ltd., U.K.) were used. The cells were fixed in cold acetone. From the tumors derived from these cells after injection into syngeneic rats, frozen as well as paraffin sections were prepared. The latter were mounted on slides coated with 0.01% poly-L-lysine [8], dewaxed and treated for 30 min with 0.1% trypsin at 37° C. Both the sections and the cell smears were then incubated for 30 min with 1 : 20 diluted normal goat serum (Nordic) to reduce the unspecific binding. They were then incubated for 60 min with rabbit antiserum against Factor VIII-related antigen (Calbiochem, Behring Corp.) or with normal rabbit serum diluted 1 : 50. After three washes in PBS the cells and sections were stained for 30 min with 1 : 40 diluted fluorescein-conjugated goat anti-rabbit Ig. Excess conjugate was removed by three washes in PBS. The slides were then mounted in buffered glycerol and examined with a Leitz Orthoplan fluorescence microscope fitted with a Ploem illuminator.

RESULTS

Histology of organ cultures

The histological examination of serial sections made from visceral yolk sac explants after 3 weeks in organ culture showed that the general structure of the membrane had been well preserved. There was a slight increase of the endodermal and mesodermal cells, a few poorly differentiated cells and well differentiated tissues like squamous epithelium and giant trophoblast cells. These observations do not differ from those described previously [5] and were similar in the polyoma-infected and control visceral yolk sac explants. However, in five out of 13 organ cultures infected with the virus a marked proliferation of mesodermal cells was also observed (Table 1). They formed foci composed of elongated (Fig. 1) or round (Fig. 2) cells with large nuclei. Mitotic figures were frequently observed (Fig. 3). In some organ cultures several foci were observed in the same section (Fig. 4). None of the control visceral yolk sac explants (Table 1) displayed these changes.

In the explants of 12-day old embryos kept for 3 weeks in organ culture, proliferation of endodermal, mesenchymal and neural tissue was observed together with patchy necrosis. No transformation and no histological differences between the virus-infected and control explants were recorded. The explants of fetal gut, kidney, cartilage and skin were nearly completely necrotized after 3 weeks in organ culture in both groups (virus-infected and control).

Tissue culture

From the 16 polyoma-infected organ cultures

*Table 1. Effect of polyoma virus on rat tissue explants kept in organ culture**

Type of explant	Histology		Cell monolayers (TC)		Implants in R/A rats	
	Number examined	Transformation	Number examined	Continuous cell lines	Number of rats	Number of tumors
Visceral yolk sac + polyoma virus	13	5	16	3	5	1
Visceral yolk sac control	15	0	17	0	8	0
Embryo + polyoma virus	7	0	11	0	9	0
Embryo control	6	0	13	0	4	0
Fetal skin, gut, kidney or cartilage + polyoma virus	16	0	5	0	10	0
Fetal skin, gut, kidney or cartilage + polyoma virus	16	0	7	0	8	0

*After 3 weeks in organ culture the explants were either examined histologically, put in tissue culture or implanted.

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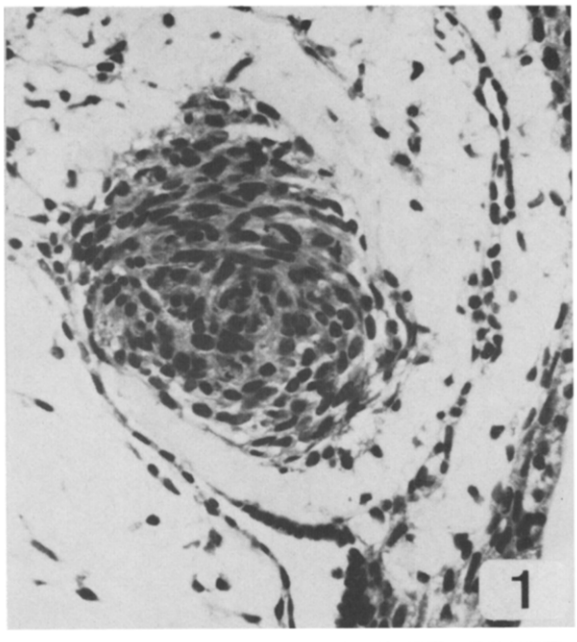
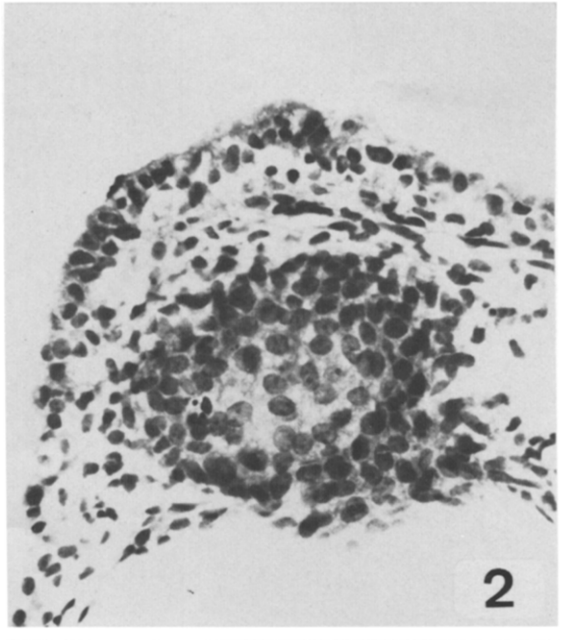




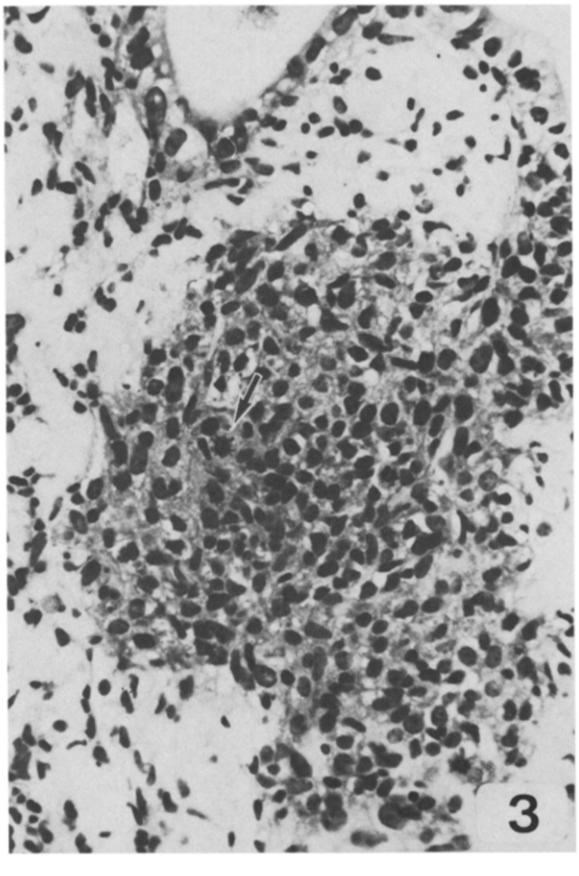
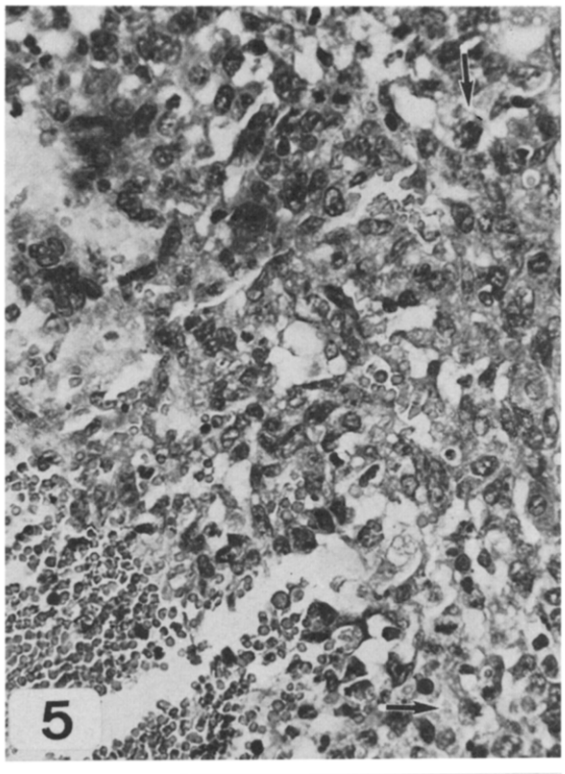


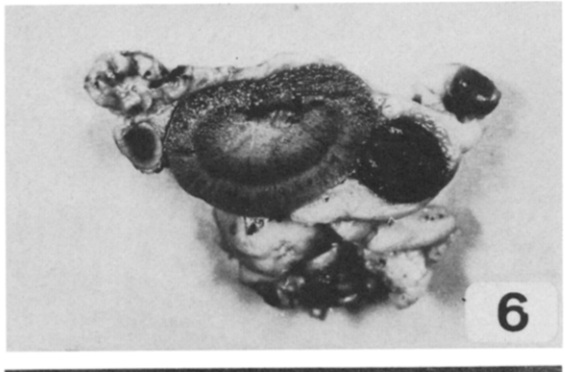
Fig. 1. *Visceral yolk sac in organ culture* 21 *days after polyoma virus infection. Focus of proliferating elongated cells. H and E,* × 660.

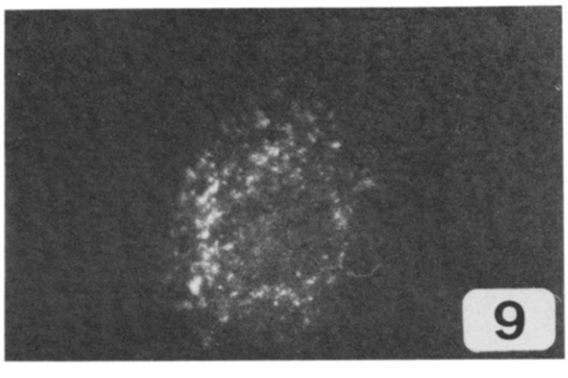
Fig. 2. *Visceral yolk sac in organ culture* 21 *days after polyoma virus infection. Focus of proliferating round cells. H and E,* × 660.

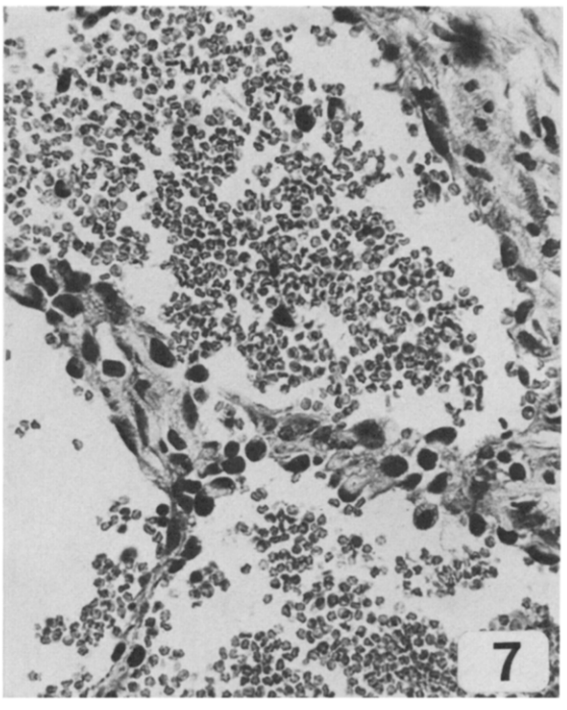
Fig. 3. *Visceral yolk sac in organ culture* 21 *days after polyoma virus infection. Proliferating cells with mitotic figures (arrow) are seen. H and E,* × 660.

Fig. 4. *Visceral yolk sac in organ culture* 21 *days after polyoma virus infection. Two foci of proliferating mesenchymal cells are observed. H and E,* × 160.









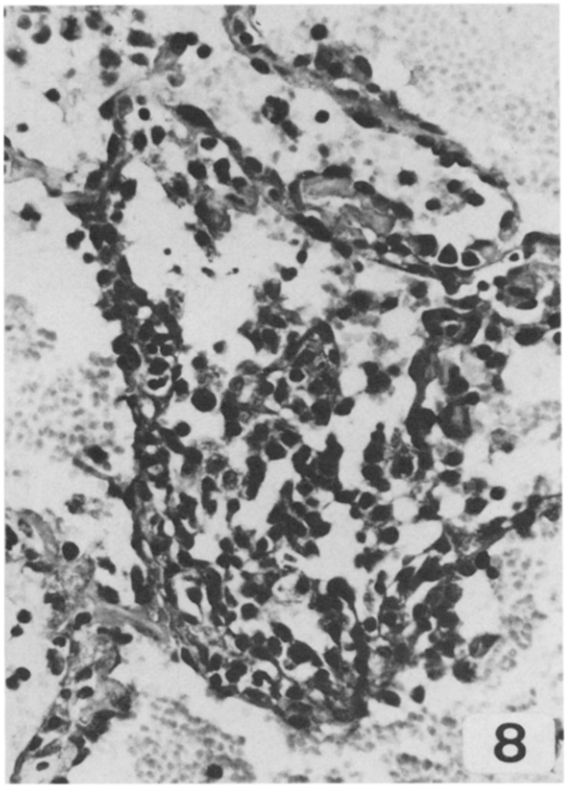


Fig. 5. Histology of hemorrhagic tumor obtained after injection of PV_2T cell line. Proliferating endothelial cells with mitoses (arrows) and blood cells are seen. H and E, × 160.

Fig. 6. Macroscopic appearance of hemorrhagic tumor obtained 4 weeks after transplantation of polyoma virus-infected visceral yolk sac grown for 21 days in organ culture.

Fig. 7. Histology of tumor shown in Fig. 6. Endothelial cells lining cavernae are observed. H and E, × 660.

Fig. 8. Histology of tumor shown on Fig. 6. Proliferation of endothelial cells is seen. H and E, × 660.

Fig. 9. Indirect immunofluorescence on PV_2T cells with antiserum to Factor VIII-related antigen. Granular cytoplasmic reaction is seen. × 1.400.

put in tissue culture dishes, three gave rise to continuous growing cell lines ($PV_{1,2,3}$). These cells grew out very rapidly from the organ culture transferred to culture dishes and gave readily rise after trypsinization to continuous cell lines. Morphologically they had a fusiform, fibroblast-like aspect. Subcutaneous injection of 5×10^6 of these cells in 3-week old female R/A rats gave rise to hemorrhagic tumors at the site of inoculation. These hemorrhagic tumors were composed of highly proliferating endothelial cells bordering lacunae filled with blood (Fig. 5). They were diagnosed histologically as hemangiosarcomas. The organ cultures from the control visceral yolk sac (not infected with polyoma virus) put in tissue culture dishes displayed outgrowth of a few epithelial-like cells. The outgrowing cells could not be maintained, however, and they necrotized progressively. The other organ cultures from embryonal or fetal origin, whether infected or not with polyoma virus, gave no consistent outgrowth of cells and when some cells grew out from the explant they did not survive for more than one week.

Transplantations

After grafting under the kidney capsule of R/A rats, one in five of the animals developed a large hemorrhagic tumor at the site of implantation of the visceral yolk sac organ culture infected with polyoma virus (Fig. 6, Table 1). The tumor consisted of cavernae filled with blood and lined by endothelial cells (Fig. 7). In some places the proliferation of the endothelial cells was quite pronounced (Fig. 8). This tumor was similar to the hemangiomas obtained after implantation in the abdominal cavity of visceral yolk sac infected by polyoma virus just prior to grafting [1]. In the four other rats grafted with polyoma virus-infected visceral yolk sac organ cultures as well as in the animals which received control visceral yolk sac organ cultures no living tissue was recorded at the implantation site 4 weeks after grafting. At the implantation site of grafted organ cultures from 12-day old embryos white nodules were observed in both the polyoma virus group and the control group. These nodules were composed of various well differentiated tissues but no evidence of tumor formation was recorded. In most cases the fetal grafts of gut, skin, cartilage and kidney were completely necrotized. Only in two cases did the gut grafts give rise to a cyst lined by gut epithelium.

Polyoma tumor specific transplantation antigen (TSTA)

The presence of polyoma TSTA on tumor cells obtained from transformed visceral yolk sac in organ culture and grown afterwards as monolayer (PV_2) was demonstrated in recipient 9-week old virgin rats after 3 weekly preimmunization with 2×10^9 PFU polyoma virus or with 2×10^7 allogenic polyoma virus-induced kidney sarcoma cells (BN/PNS). As shown on Fig. 10 the preimmunization markedly decreased the incidence and the growth of tumors obtained after subcutaneous inoculation of 10^5 PV_2 cells. In the control group, pre-immunized with 2×10^7 allogenic cells of a carcinogen-induced fibrosarcoma (BN/DMBA) all rats developed large tumors during the 13-day observation period.

Factor VIII-related antigen

The immunofluorescent studies showed a positive reaction with the rabbit antiserum against Factor VIII-related antigen in the cytoplasm of about 50% cultured hemangiosarcoma cells (Fig. 9), while other tumor lines like yolk sac carcinoma (F40) or kidney sarcoma (PNS) were completely negative. Similar results were observed on cryostat and paraffin sections. The endothelium of blood vessels was positive in all kinds of tumor. If the antiserum was replaced by normal rabbit serum, no fluorescence was seen.

DISCUSSION

The results of these experiments confirm our hypothesis that in rat the development of vascular tumors is due to the particular tropism of polyoma virus for endothelial cells. Indeed, from the different tissue components present in the rat visceral yolk sac, only the endothelial cells were transformed. This phenomenon, initially observed *in vivo* [2, 5], is now confirmed by our *in vitro* results.

A similar sensitivity of a particular cell type for polyoma virus has been demonstrated for the mouse salivary gland. The epithelial cells of this gland are indeed particularly sensitive to the oncogenic activity of the polyoma virus both *in vivo* and in organ culture [9, 10]. Organ cultures of rat salivary glands, however, were found by the same authors to be completely resistant to the oncogenic activity of the polyoma virus [11].

Although morphologically the foci of proliferating cells observed in cultured yolk sac could not be distinguished from other mesodermal cells, their endothelial nature could be shown (i) by the grafting of cultured polyoma virus-infected visceral yolk sacs under the kidney capsule which gave rise to vascular tumors at the site of implantation, and (ii) by the subcutaneous injection of cultured cells obtained from polyoma virus-infected visceral yolk sac organ cultures. These *in vitro* cultured cells always gave rise to hemangiosarcomas when reinjected into syngeneic rats. The endothelial nature of these tumors was not only revealed by their morphological characteristics but also by the detection of Factor VIII-related antigen in the tumor cells. This antigen is specific for endothelial cells

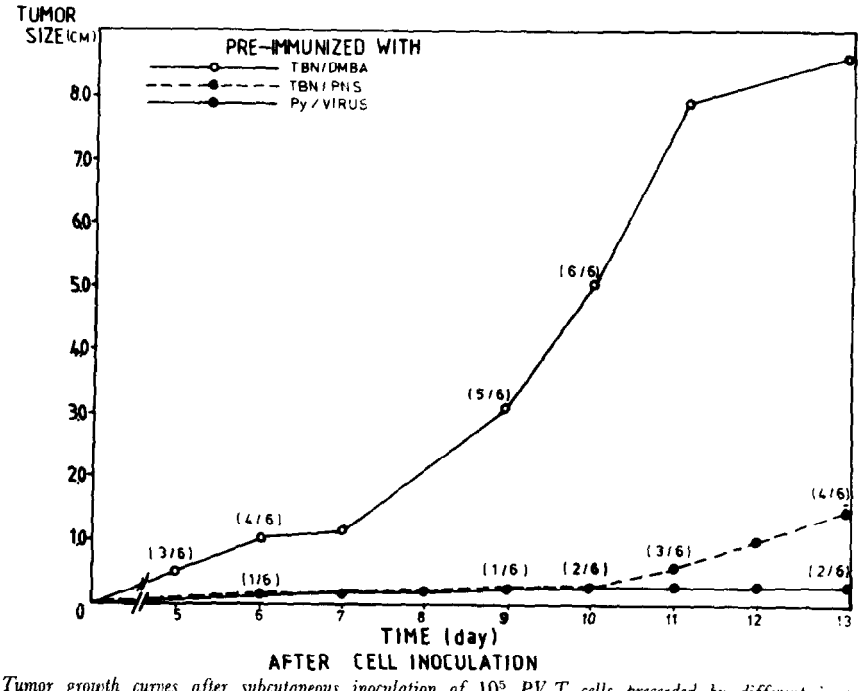


Fig. 10. Tumor growth curves after subcutaneous inoculation of 10^5 PV_2T cells preceeded by different immunization. (/) = number of tumors/number of rats injected.

and megakaryocytes [12, 13] and is used for the diagnosis of tumors of vascular origin [14]. That the endothelial cells were transformed by the polyoma virus was proven by the detection of polyoma-specific TSTA on the PV_2 cell line derived from a virus-infected visceral yolk sac organ culture. Moreover, the same cell line was shown to express the specific polyoma T-antigens (large, middle and small). The latter data were kindly provided by Dr. G. Meyer (INSERM, Marseille, France). Although in organ cultures of visceral yolk sac proliferation of epithelial, mesenchymal and less-defined poorly differentiated cells is observed [5], only the endothelial cells are apparently highly sensitive for the transformation capacity of polyoma virus.

A similar sensitivity was observed in young or adult immunosuppressed rats injected with polyoma virus. Most of these animals developed hemangiomas generally located in the brain [4, 15]. The possibility to induce polyoma tumors in immunodeficient adult animals [16] and in immunologically privileged sites (brain) indicate that the cellular immunity plays a role in the immune surveillance of polyoma tumor outgrowth. The immunogenicity of malignant polyoma tumors is well documented. It is essentially due to the presence on the cell membrane of TSTA which are believed to play a major role in host resistance to cancer [17, 18, 19]. These antigens have been demonstrated on the cells of yolk sac-derived malignant hemangiosarcomas [2] and of benign hemangiomas developed from ectopic implanted yolk sac membrane after *in vitro* polyoma virus infection [5]. They were also found to be present in the vascular tumors obtained in the present experiment.

It is interesting to note that the visceral yolk sac infected with the virus *in vitro* and grafted immediately afterwards *in vivo* undergoes only benign transformation (hemangiomas) [1], whereas after previous passage in organ culture malignant transformation may also be observed. The immunological reaction of the host cannot explain this phenomenon since both the benign and malignant tumors express the polyoma TSTA. Another explanation may be found in the theory of Pierce [20] and of Pierce and Cox [21] who state that the stage of differentiation of the target stem cell at the time of the action of a carcinogen will determine whether or not a tumor is benign or malignant. If, during cell renewal, a carcinogen affects undifferentiated cells, a malignant tumor will result; if the same agent affects more differentiated cells, capable of one more division, a benign tumor develops. It is possible that the special conditions used in the present experiment like precultivation of visceral yolk sac in a roller system before organ culture and the use of a rich medium favors the multiplication of endothelial cells, as was shown for other tissues of the membrane [5]. Such intensive proliferation has indeed been demonstrated in the displaced visceral yolk sac after fetectomy [22] but not in the ectopic transplanted pieces of this membrane (unpublished results).

Hence, the possibility should be considered that

the virus transforms the fast proliferating cells in the organ culture which are less differentiated and therefore develop into hemangiosarcoma, while the more slowly dividing cells in the ectopic transplanted yolk sac undergo only benign proliferation. A similar phenomenon may take place after fetectomy where the injected polyoma virus transforms angioblastic cells, which are present in the displaced visceral yolk sac at various stages of differentiation. Dependent upon the stage of differentiation at the time of viral induced transformation, the transformed cells will either develop into benign hemangiomas or into malignant hemangiomas [2]. This explanation seems also probable considering the results of experiments performed in rats *in vivo* [2]. Moreover, the particular sensitivity of endothelial cells for polyoma virus transformation does not depend on a better exposure to the virus of the cells lining the blood vessels since in organ culture where polyoma virus can easily penetrate into all types of cells, only the endothelium is transformed. The other tissues, which also proliferate under these conditions are not transformed by the virus and they differentiate to the same extent as in control cultures. The latter observation indicates that polyoma virus does not influence the differentiation capacity of the visceral yolk sac *per se*.

The failure to detect transformation of the vascular endothelium in fragments of embryos maintained in organ culture compared to the positive results obtained with ectopic grafts of embryos previously infected with the virus [1] deserves further investigation.

Since the rat visceral yolk sac displays in organ culture both the capacity to proliferate and differentiate and to be transformed by an oncogenic virus, it may represent an interesting alternative model for the mouse salivary gland [11] and kidney [23] which have been extensively studied for their sensitivity *in vitro* to the transforming capacity of polyoma virus.

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REFERENCES

1. Lu YL, Sobis H, Van Hove L, Vandeputte M. Polyoma virus-induced hemangiomas in grafts of visceral yolk sac and of embryos. *Eur J Cancer Clin Oncol* 1985, **21**, 631–636.
2. Vandeputte M, Meyer G, Sobis H. Vascular tumors induced by polyoma virus in pregnant rats. *J Natl Caner Inst* 1976, **56**, 517–521.
3. Eddy BE, Stewart SE, Stanton MF, Marcotte JM. Induction of tumors in rats by tissue-culture preparations of SE polyoma virus. *J Natl Cancer Inst* 1959, **22**, 161–171.
4. Vandeputte M, Denys P, Leyten R, De Somer P. The oncogenic activity of the polyoma virus in thymectomized rats. *Life Sci* 1963, **2**, 475–478.
5. Lu YL, Sobis H, Van Hove L, Vandeputte M. Differentiation potentiality of rat visceral yolk sac in organ culture. *J Embryol Exp Morphol* 1984, **80**, 127–136.
6. Crawford LV. The adsorption of polyoma virus. *Virology* 1962, **18**, 177–181.
7. Dulbecco R, Freeman G. Plaque production by the polyoma virus. *Virology* 1959, **8**, 396–397.
8. Van Noorden S, Polak JM. Immunocytochemistry today. Techniques and Practice. In: Polak JM, Van Noorden S, eds. *Immunocytochemistry, Practical Applications in Pathology and Biology*. Wright PSG, Bristol, London, Boston, 1983, pp. 11–42.
9. Dawe CJ, Law LW. Morphological changes in salivary gland tissue of the newborn mouse exposed to parotid-tumor agent *in vitro*. *J Natl Cancer Inst* 1959, **23**, 1157–1177.
10. Dawe CJ. Cell sensitivity and specificity of response to polyoma virus. *Nat Cancer Inst Monogr* 1960, **4**, 67–128.
11. Dawe CJ, Law LW, Rowe WP. Influence of age, species and immune factors on the response of salivary gland to polyoma virus in tissue culture. *Pathol Biol* 1961, **9**, 711–718.
12. Jaffe EA. Synthesis of factor VIII by endothelial cells. *Ann NY Acad Sci* 1982, **401**, 163–170.
13. Shearn SAM, Peake IR, Giddings JC, Humphrys J, Bloom AL. The characterization and synthesis of antigens related to Factor VIII in vascular endothelium. *Thromb Res* 1977, **11**, 43–56.
14. Sehested M, Hou-Jensen K. Factor VIII related antigen as an endothelial cell marker in benign and malignant diseases. *Virchows Arch (Path Anat)* 1981, **391**, 217–225.
15. Vandeputte M. Antilymphocytic serum and polyoma oncogenesis in rats. *Transplant Proc* 1969, **1**, 100–105.
16. Vandeputte M, Eyssen H, Sobis H, De Somer P. Induction of polyoma tumors in athymic nude mice. *Int J Cancer* 1974, **14**, 445–450.
17. Meyer G. Polyoma virus as a model for viral skin carcinogenesis. *Bull Cancer* 1978, **65**, 145–150.
18. Steuer AF, Ting RCY. Immune response to polyoma- and adenovirus-induced tumors. In: Blasecki JW, ed. *Mechanisms of Immunity to Virus-Induced Tumors*. New York, Basel, Marcel Dekker Inc., 1981, p. 287.

19. Vandeputte M. Immunopathology of polyoma-induced tumors. *Springer Sem Immunopath* 1982, **4**, 317–332.
20. Pierce GB. Neoplasms, differentiations and mutations. *Am J Pathol* 1974, **77**, 103–114.
21. Pierce GB, Cox WF. Neoplasm as caricatures of tissue renewal. In: Saunders GF, ed. *Cell Differentiation and Neoplasia*, New York, Raven Press, 1978, pp. 57–66.
22. Sobis H, Van Hove L. Vandeputte M. Cellular events during early formation of yolk sac derived teratomas.*J Embryol Exp Morphol* 1982, **70,** 225–240.
23. Vainio T, Saxen L, Toivonen S. The acquisition of cellular resistance to polyoma during embryonic differentiation. *Virology* 1963, **20**, 380–385.