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Experimental Rat Model for Human Yolk Sac Tumor

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Abstract—*The morphological and biological characteristics of experimentally induced rat yolk sac carcinomas (ysca) are compared to those of human yolk sac tumors. It is shown that the rat ysca shares many morphological and biological properties with its human counterpart although the cellular origin is probably different. Whereas the human yolk sac tumors are believed to be of germ cell origin, the rat visceral yolk sac-derived tumors are not. The hypothesis is formulated that the rat ysca are derived from multipotential cells different from germ cells, and which originate in the extra-embryonic membrane after displacement.*

INTRODUCTION

HUMAN yolk sac tumor is a malignant neoplasm of testis and ovary or, rarely, of other organs. After comparative morphological studies Teilum [1] showed that the typical structures of this tumor correspond to the Duval sinuses in the rat placenta. These sinuses represent the invaginations of the visceral and the parietal yolk sac into the rat placenta. They are lined by a single layer of flat cells within the middle cores of vascular mesoderm surrounded by columnar epithelium. As was shown later the endodermal patterns of human yolk sac tumor secrete alpha-fetoprotein (AFP) [2, 3] and hyaline [4], the characteristic features of, respectively, visceral and parietal yolk sac elements in rodents. An experimental model for the human tumor is rat yolk sac carcinoma (ysca). Indeed, this rat tumor presents many similarities with the human neoplasm since it is composed of parietal as well as visceral endodermal cells [5] and has been lately also found to display mesenchymal and trophoblastic structures [6].

HISTORY OF THE EXPERIMENTAL YOLK SAC CARCINOMA (YSCA)

The experimental induction of yolk sac carcinomas was initially described in our laboratory in rats which were fetectomized at day 12 of pregnancy and injected with mouse sarcoma virus (MSV) in the placentas after displacement of the visceral yolk sac [7]. These rats developed tumors (ysca) 2–3 months after operation. Afterwards it was shown by Sakashita *et al.* [8] and ourselves [9] that displacement of the visceral yolk sac outside the uterus of fetectomized animals without MSV inoculation also led to the development of ysca. In the latter model the latency period was found to be much longer (4–10 months) and the tumor incidence lower. Rat ysca develops also after implantation of 8–9-day-old embryo under the kidney capsule of a syngeneic host [10]. The tumors induced by all these methods infiltrate the organ in which they develop and metastasize mostly into the peritoneum, the lymph nodes, the ovaries and eventually into the lungs. The tumors are transplantable in syngeneic hosts. Both the primary and the transplantable tumors display the visceral yolk sac pattern and the parietal yolk sac pattern (biphasic). The visceral yolk sac cells secrete alpha-fetoprotein (AF) [11] and express the yolk sac antigen 1 [12]. The parietal cells produce hyaline and react with monclonal antibodies directed against yolk sac antigen 2 [13].

There are, however, differences between the embryo-derived and the visceral yolk sac-derived yscas. While the embryo-derived yscas are composed only of visceral and parietal endodermal cells, in the yolk sac-derived tumors trophoblast cells and mesenchymal cells are also observed [6]. Sometimes the formation of structures very similar to the Schiller–Duval bodies present in human tumors is also found. The differences in morphology between both types of rat ysca can be explained by a difference in the development stage of the tissue from which the tumor originated. The extraembryonic part of 9-day-old egg cylinder from which the ysca develops [14] is composed of young endoderm and

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mesoderm in which blood vessels are not yet formed. It is therefore possible that, after transplantation, the mesenchyma differentiates poorly (or not) whilst the endoderm proliferates to form biphasic endodermal ysca. On the contrary the 12 day old visceral yolk sac contains endoderm and mesoderm with well formed and numerous blood vessels. Moreover, after displacement of the membrane, proliferation of the mesodermal and endodermal cells is observed [15]. This proliferation is accompanied by the appearance of numerous poorly differentiated cells [16] (*vide infra*). Their presence also explains the formation in the tumor of complex structures similar to endodermal sinuses or Schiller–Duval bodies, which thereby make the experimental neoplasm more similar to its human counterpart. The mesenchymal tissue in the ysca may induce trophoblast differentiation. Such an inductive property of mesodermal tissue on the differentiation of trophoblast is known during embryogenesis [17–19]. In the embryo-derived ysca such mesenchyma was never found. Therefore the inductive properties of mesenchymal cells seem to form the basis for the differences in morphology found between embryo-derived and visceral yolk sac-derived ysca.

THE CELLULAR ORIGIN OF RAT YOLK SAC CARCINOMAS

As stated before, experimental rat ysca can develop from the extraembryonic portion of the egg cylinder [14] or from the visceral yolk sac displaced at day 12 of pregnancy. It is important to notice that in the yolk sac-derived primary ysca, apart from the malignant cells described above, various well-differentiated tissues are also observed [20]. These well-differentiated tissues are already detectable less than 1 month after the operation, and develop into benign teratomas outside the uterus at the place where the visceral yolk sac was displaced after fetectomy [21]. They are present well before the malignant yscas are observed and their development is not dependent upon virus (MSV) inoculation. These data show that the visceral yolk sac pulled outside the uterus can give rise to two kinds of tumor: benign teratomas and malignant yscas. A similar observation was made in the hamster [22] and the mouse [23]. Also in these species the displacement of the visceral yolk sac after fetectomy induces the development of benign teratomas in all animals with the eventual appearance of yolk sac carcinomas at a later stage.

The nature of the stem cells from which the malignant (ysca) and benign tumors (teratomas) originate is not known with certainty. Since they give rise to a variety of tissues derived from all germ layers, these stem cells must however be multipotential. As teratoma and yolk sac tumor in humans are considered to be of germ cell origin, experiments were performed to verify whether these tumors in rodents have a similar origin. The following experimental data indicate that this is not the case. It was first shown that rats treated with Busulphan, which destroys the germ cells during their migratory phase in embryogenesis, develop as many teratomas [24] and yolk sac carcinomas as the non-treated control animals [25]. Afterwards we observed in 129 Sv-S1 mice that the development of teratomas [26] and of yolk sac carcinoma [27] from the displaced visceral yolk sac from genetically sterile embryos, deficient in primordial germ cells, was identical to that recorded from heterozygotes and from genetically normal embryos.

As the rat visceral yolk sac also proliferates and differentiates in organ culture *in vitro* [28] the possible presence of germ cells was looked for using alkaline phosphatase as a marker. It was shown that these cells are absent in the membranes examined before as well as after organ culture [29].

From these *in vivo* and *in vitro* experiments we conclude that yolk sac-derived benign and malignant tumors are not of germ cell origin. Therefore one has to postulate the appearance of multipotential cells other than germ cells in the displaced visceral yolk sac and which give rise to the various structures found in these benign teratomas. Hence, the possibility of transdifferentiation has to be considered. In order to reach such a conclusion one has, however, to verify the morphological changes that appear at the cellular level in the earlier stages of tumor formation. Sequential morphological studies performed after the displacement of the visceral yolk sac showed the proliferation of mesodermal and endodermal cells [15] and the appearance of poorly differentiated cells in the fetal membrane left a few days outside the uterus *in vivo* [16] or kept in organ culture *in vitro* [28]. These poorly differentiated cells can apparently differentiate into various tissues of mesodermal, endodermal and ectodermal origin to form teratomas or can undergo malignant transformation and develop into yscas. Although we do not known for certain whether these poorly differentiated cells are of endodermal or mesodermal origin, preliminary experiments indicate that they dedifferentiate from endodermal cells and then redifferentiate into various tissues. Such potentiality of single endodermal cells to differentiate into visceral and parietal endoderm, mesoblast and trophoblast was demonstrated by cloning experiments with yolk sac carcinoma cells [30].

VIRAL VERSUS NON-VIRAL INDUCED RAT YOLK SAC CARCINOMAS

As mentioned before, rat ysca can be induced by displacement of the visceral yolk sac after fetectomy or by the same technique followed by the injection into the placenta of Moloney murine sarcoma virus

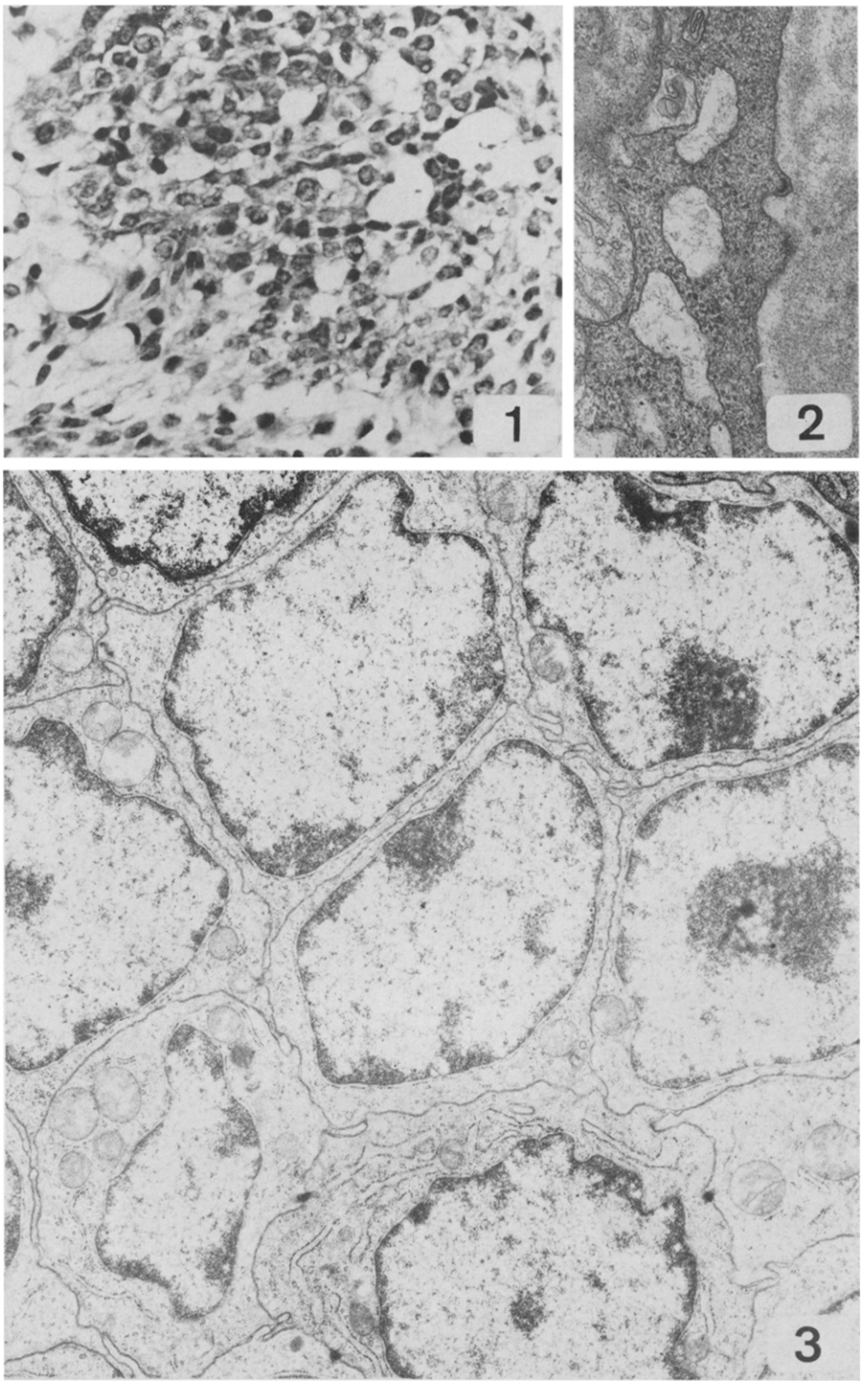
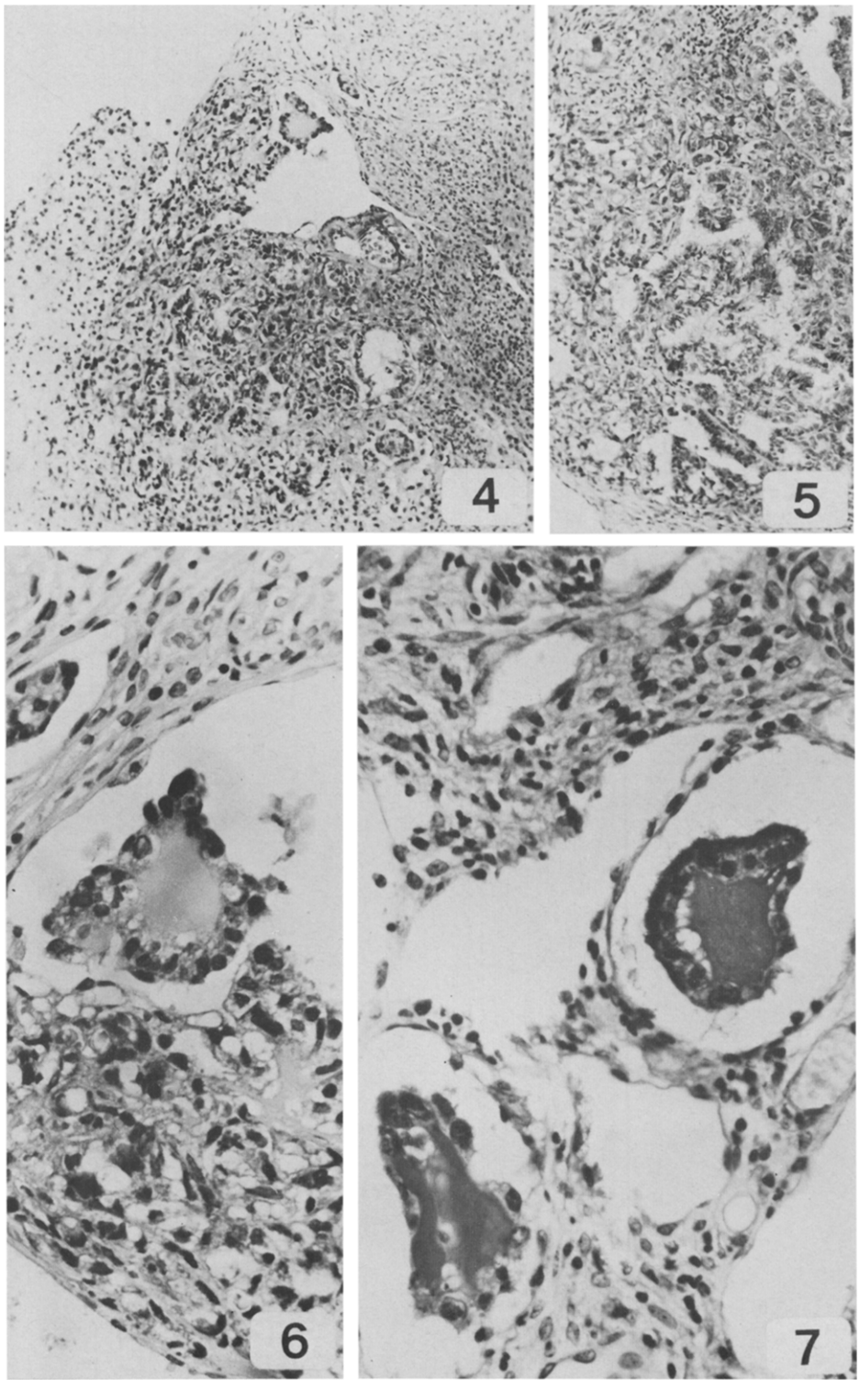


Fig. 1. *Focus of less differentiated cells in the displaced visceral yolk sac* 6 *days after fetectomy. H. and E.* × 660.

Fig. 2. *Cell of the visceral yolk sac* 3 *days after displacement and MSV-inoculation. Two budding C-type particles are seen. TEM* × 20,000.

Fig. 3. *Focus of poorly differentiated cells* 9 *days after displacement of the visceral yolk sac. TEM* × 4500.



Figs. 4,5. *Foci of yolk sac carcinoma* 15 *days after MSV injection. H. and E.* × 180.

Fig. 6. *Embryoid-like body next to focus of yolk sac carcinoma. H. and E.* × 660.

Fig. 7. *Embryoid-like bodies in blood vessels of contralateral horn. PAS* × 660.

(Mo-MSV). The morphological and antigenic characteristics of the viral induced and non-viral induced ysca are similar [31]. Moreover, both kinds of tumor transplanted into syngeneic rats metastasize to the regional lymph nodes, into the peritoneal cavity and, sometimes, into the lungs. Yet, some definite differences between both groups exist which clearly indicate that, at least in the rat inbred R/A strain, MSV inoculation plays a definite role in the induction of visceral yolk sac derived malignant tumors. This conclusion is based upon the following findings: first, the tumor incidence is significantly higher in the MSV-inoculated group (80% vs. 42%); second, the latency period is markedly shortened (average of 10 weeks vs. 23 weeks for the non-virus group); third, all the early tumors (latency period of less than 3 months) were only recorded in the MSV-inoculated horn and never in the contralateral horn where the displacement of the visceral yolk sac was not followed by virus inoculation; fourth, in rats preimmunized subcutaneously with MSV 3–4 weeks before displacement of the visceral yolk sac followed by intraplacental injection of the virus, the results were quite similar to those recorded in the non-virus group; and fifth, only in the MSV-injected group did we observe the development of a few less differentiated tumors tentatively diagnosed as embryonal carcinomas [31].

Since preimmunization with the virus led to protection of the host against the development of early tumors in the MSV-treated group it is likely that the host immune response plays a role in the early events of viral transformation. Hence, the possibility that at the initial stages of tumor development some or most of the transformed cells are virus producers which by a process of immune selection are rejected to yield a population of non-producer cells as is found in the yolk sac carcinomas. In order to verify this hypothesis and to verify whether the shorter latency period in the viral induced ysca is related to the earlier appearance of malignant transformation, a sequential morphological study was made of the displaced visceral yolk sac in rats inoculated or not with MSV into the placentas. For this study 36 inbred R/A rats were fetectomized at day 12 of pregnancy and the visceral yolk sac exteriorized (displaced) through the incision. Half of the rats (group I—18 rats) were inoculated in one uterine horn with Moloney MSV. The contralateral horn (control) was injected with PBS. The other 18 rats (group II) were not injected with Mo-MSV. At regular intervals (every 3 days) two rats of each group were killed and serial sections cut for histological and ultrastructural examination of the displaced visceral yolk sac attached to the uterine wall.

The histology of the displaced visceral yolk sacs from rats killed at day 3 and 6 after fetectomy was similar in both groups, with or without virus inoculation. Proliferation of endodermal and mesodermal cells and the appearance of less differentiated cells was observed in all rats (Fig. 1). At the ultrastructural level C type particles were found in a few cells in five out of eight displaced visceral yolk sacs. These C type particles were only seen in the MSV-inoculated animals (Fig. 2). They were no longer observed at the later stages.

Starting from 9 days after the operation the progressive development of teratomas was observed similar to that described previously [15]. These benign tumors composed of various well-differentiated tissues were similar in the virus-inoculated and the contralateral horns in MSV-treated rats as well as in the control animals. In one rat of group I killed 9 days after fetectomy a small focus of proliferating poorly differentiated cells displaying morphological signs of malignancy was found in the MSV-inoculated uterine horn. The cells had big nuclei and a cytoplasm rich in ribosomes but few other organelles (Fig. 3). Mitoses were present but no C type particles were observed. In another rat autopsied 15 days after operation two foci composed of typical yolk sac carcinoma cells were found (Figs. 4, 5). They were located in two different places where the visceral yolk sacs were outside the virus-injected uterus. Moreover, some embryoid-like bodies were present near the tumors (Fig. 6). In the contralateral horn foci of carcinoma cells were seen in blood vessels (Fig. 7). In the rats killed at later stages one to four small yolk sac carcinomas were observed in the MSV-treated uterine horn. Metastases consisting of embryoid-like bodies or groups of carcinoma cells were observed in the contralateral horn. They were always located in the blood vessels. All these data are summarized in Table 1.

The present study clearly indicates that Mo-MSV facilitates the malignant transformation of visceral yolk sac cells. As early as 9 days after virus inoculation a focus of tumor cells was observed. The number and volume of these malignant foci increased gradually afterwards. In contrast, no morphological signs of malignant transformation were recorded in the displaced membrane of animals which were not inoculated with MSV. This indicates that the virus accelerates the transformation and explains the shorter latency period previously recorded in the virus treated group. Microscopically, metastases started to appear as early as 15 days after virus inoculation which indicates that the tumor cells proliferate and enter the circulation at an early stage. From this study one cannot decide, however, whether virus inoculation causes the higher incidence and shorter latency period of ysca by acting as a transforming agent on the yolk sac cells or in an indirect way, e.g. by influencing the host immune response. To approach this problem

Table 1. Sequential appearance of malignant transformation in displaced visceral yolk sac

Group		Days after fetectomy									
		3	6	9	12	15	18	21	24	27	Total
I.	MSV inoculated (n = 18)										
	Ia. virus injected horn	0/8*	0/8	1/8	0/8	2/8	2/8	1/7	2/7	4/8	12/70
	Ib. contralateral horn	0/9	0/7	0/8	0/7	2†/7	0/6	0/6	1†/8	3†/9	6/65
II.	No virus (n = 18)	0/22	0/16	0/23	0/22	0/15	0/23	0/12	0/15	0/18	0/166

Two rats were examined every 3 days.
*No. of foci of transformation/No. of fetectomies.
†Metastases.

we looked for the presence and expression of *mos* sequences in spontaneous and in Mo-MSV induced rat ysca. While c-*mos* was found in all yscas tested, only in the viral induced tumors were additional *mos*-sequences recorded. The latter were identified as randomly integrated Mo-MSV provirus [32]. In none of the yscas induced by displacement alone (spontaneous) were these v-*mos* sequences observed. All these tumors developed much later than those which contained MSV proviruses.

These data indicate that the presence of integrated Mo-MSV proviruses in the cell genome facilitates the oncogenesis in this system. Significant amounts of *mos*-related RNA (v-*mos* or c-*mos* transcripts) were not found, however, in any of yolk sac carcinoma derived cell lines [32]. The c-*mos* gene is known to be transcriptionally silent [33] in normal adult cells, but Propst and Vande Woude [34] demonstrated expression of the gene in mouse testes, ovaries, early embryos and epididymis. It is possible that c-*mos* plays a role in differentiation and that v-*mos* can block the differentiation as has been shown in an *in vitro* system [35]. Rat thyroid epithelial cells transformed by retroviruses produced viral particles of both the leukomogenic helper and the sarcomogenic pseudotype. In these cells an elevated level of v-*mos* RNA was found [35].

Although no virus production was detected in the viral-induced yscas, it is likely that at the initial stages of tumor outgrowth, virus producing cells are present since the presence of C type particles was regularly observed at the early stages of transformation (*cf.* above). It seems probable that at this stage of tumor development the v-*mos* is expressed (experiment in progress). These virus producing and immunogenic cells are likely to be eliminated by immune selection so that only the non-virus producer cells grow further in the host. This hypothesis is strengthened by the observation that after conversion into a producer line of rat Mo-MSV induced embryonal carcinoma cells by superinfection with an endogenous C-type mouse virus, these cells no longer grew in syngeneic rats [36]. When, however, this producer line was passaged into nude mice only non-producer cells were selected. The latter produced tumors in the syngeneic rats. In the producer cells an elevated level of v-*mos* RNA was found [32].

RAT VERSUS HUMAN YOLK SAC CARCINOMA

The experimental rat yolk sac carcinoma presents many morphological and biological similarities with human yolk sac tumor. Both neoplasms are very malignant and are composed of visceral and parietal yolk sac elements eventually intermingled with mesenchymal and trophoblastic structures. The parietal yolk sac elements secrete high amounts of hyaline, the visceral secrete alpha-fetoprotein (AFP). The latter is considered a good serological marker for the tumor since high serum levels are detected both in the human and in the rat [37, 38]. The high serum levels of AFP disappear after treatment of the tumor and reappear when metastases or recurrences develop. We found, however, that after multiple *in vivo* transplantations the AFP serum level may eventually fall to normal levels in the rat (unpublished results). This may be explained by either a decrease or loss in the AFP-secreting capacity of the tumor cells or by a gradual overgrowth of cells of the parietal type which secrete hyalin instead of AFP. Eventually pure parietal endodermal tumor cells can even be obtained as shown *in vitro* by Wewer [39]. In this connection one should mention the observation of Damjanov *et al.* [40] who reported a case of *in vivo* cloning of a human yolk sac tumor. A recurrence of this tumor after treatment no longer secreted AFP. Histologically the recurrence was shown to consist of pure parietal yolk sac elements whereas the primary tumor displayed a typical biphasic pattern and secreted AFP.

Although the human and the rat yolk sac tumors share many morphological and biological characteristics, their cellular origins are apparently different. Teilum [41] considered the human yolk sac tumor (endodermal sinus tumor) to be an extraembryonic

derivative of embryonal carcinoma differentiating in the yolk sac direction. Yolk sac structures can also be observed in or metastasize from teratomas [42]. In both cases, the tumors are believed to originate from germ cells. From the data previously described we conclude that the experimental rat yolk sac carcinoma is not derived from germ cells. We rather favor the hypothesis that they derive from multipotential cells that appear in the displaced yolk sac and which can give rise either to benign teratomas or, after malignant transformation, to yolk sac carcinomas. Whether these multipotential cells are of endodermal origin only is still an open question.

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