Visceral yolk sac-derived tumors

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ABSTRACT Externalization of the visceral yolk sac, after fetectomy, induces the development of extraembryonal fetal tumors in rodents. These tumors are either benign teratomas that appear 3 to 4 weeks after the displacement of the yolk sac or malignant tumors, i.e. yolk sac carcinomas. The latter appear 4 to 8 months after the surgery. If however, Mouse Sarcoma Virus (MSV) is injected in the placentas at the time of fetectomy (day 12 of pregnancy) the malignant tumors develop much earlier (2 to 3 months after surgery) and some display characteristics of embryonal carcinoma. Whether virus induced or not, the yolk sac carcinomas that develop from the displaced visceral yolk sac possess the same morphological and biological characteristics. They are composed of both parietal and visceral yolk sac structures and sometimes trophoblast. The tumors metastasize, grow in ascites form and kill their host. They are readily transplantable in syngeneic rats and grow in tissue culture as an epithelial-like sheet of cells. On the other hand, the benign teratomas are composed of various well differentiated adult tissues. In these tissues, derivatives of all three germ layers are observed. Numerous experiments prove that the stem cells for these various adult tissues are not germ cells. Instead the stem cells are multipotential cells that arise in the displaced yolk sac by a process of dedifferentiation. These poorly differentiated cells originate from the endoderm of the displaced visceral yolk sac. By redifferentiation they give rise to the various adult tissues characteristic for benign teratomas. The multipotential poorly differentiated cells are also likely to be the target cells for malignant transformation. Malignant transformation of these cells, whether induced by a virus or spontaneously occurring in the displaced yolk sac, leads not only to the development of yolk sac carcinomas and eventually embryonal carcinoma but also, although rarely, to choriocarcinoma. The latter tumor is transplantable in allogeneic hosts. It is hormonally active since it secretes lactogen and progesterone. The extraembryonal fetal tumors and in particular the rat yolk sac carcinomas and choriocarcinoma proved to be a good source for the detection of oncofetal antigens. At least two different oncofetal endodermal antigens were detected with monoclonal antibodies (mab) made after immunization with yolk sac carcinoma. Another mab, made against choriocarcinoma, was found to react specifically with the cytotrophoblast both in the normal placenta and in the tumor. No other placental cells showed a positive reaction.

KEY WORDS: *teratoma, yolk sac carcinoma, choriocarcinoma, yolk sac, dedifferentiation*

Introduction

In all pregnancies resulting from heterospecific matings, the fetus is an allogeneic graft in the uterus of the mother. Since this allograft is successful, some particular mechanism must be operative to allow its normal development. Whereas many investigators stress the importance of the trophoblast as a quarantining layer protecting the fetus against immune aggression (Vandeputte and Sobis 1972; Zuckerman and Head, 1987), others give experimental evidence indicating that the pseudopregnant or pregnant uterus may function as an immunologically privileged site (Beer and Billingham, 1974; Vandeputte and Sobis, 1975b). Whatever the mechanism involved, we reasoned that the possibility may exist that, because of this special fetal-maternal relationship, one could induce tumors in the pregnant uterus by inoculating a virus which normally does not provoke neoplasms in adult rodents (Vandeputte and Sobis, 1975a). Virus-transformed cells are indeed rejected by the mature immune system in adults before a sufficiently large tumor is formed. For these experiments we used two different oncogenic viruses: the RNA Moloney Mouse Sarcoma Virus (MSV) and the DNA Polyoma virus (Py). Both viruses only induce tumors when inoculated in newborn rats. Adult rats are completely resistant

Abbreviations used in this paper:Mo-MSV, Moloney Mouse Sarcoma Virus; Py, Polyoma Virus; AFP, alphafetoprotein; G6PD, glucose-6-phosphate dehydrogenase; mab, monoclonal antibodies.

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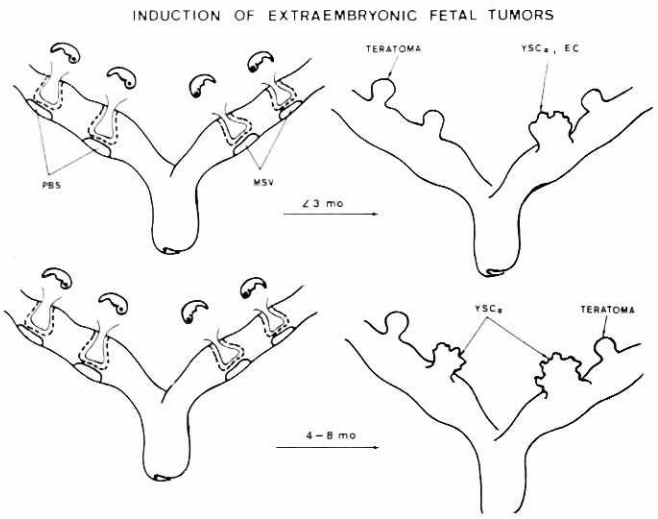


Fig. 1. Surgical procedure for the induction of tumors: —— *visceral yolk sac;* – – – – *parietal yolk sac.*

to the oncogenic activity of these viruses. However, when inoculated into the placenta of fetectomized rats, we found that they induce extraembryonal fetal tumors in the adult animal. The MSV-induced tumors were either yolk sac carcinomas or embryonal carcinomas (cf. as described below) whereas with Py virus, hemangiomas and hemangiosarcomas were induced. These vascular tumors derived from the Py virus transformed endothelial cells present in the visceral yolk sac. Since they do not differ in their morphological or immunological characteristics from those obtained after injection of the virus in neonatal rats, they will not be further discussed. The reader interested in this model is referred to the several papers published elsewhere (Vandeputte *et al.*, 1976; Lu *et al.*, 1985, 1987).

Yolk sac carcinoma

The experimental induction of yolk sac carcinoma was initially described in our laboratory in rats which were fetectomized at day 12 of pregnancy and injected with Moloney Mouse Sarcoma Virus (MSV) into the placenta of one uterine horn (Vandeputte *et al.*, 1973) (Fig. 1). About one third of the rats developed tumors 2-3 months after surgery (the animals were kept for 4 months only) The tumors were located in the virus inoculated horn. Their size varied from 0.5 to 4 cm in diameter. Metastases were very often seen in peritoneum, sometimes in lymph nodes, ovaria and in the lungs. Frequently the tumor-bearing rats also displayed a hemorrhagic ascites with numerous malignant cells. Apart from these malignant tumors, nodules of varying sizes and color attached to both the virus-infected and control uterine horn were also observed. They had a smooth surface and were found to be benign tumors composed of well differentiated tissues (cf. teratoma). On histological examination most of the macroscopic malignant tumors were composed of round or polygonal cells with pleomorphic nuclei and amphophilic cytoplasm containing PAS-positive diastase resistant granules–hyalin. Mitotic figures were numerous. The neoplastic cells formed rosettes, cords, columns or papillary structures (Fig. 2). Between single cells or groups of cells a PAS-positive substance was observed (Fig. 3). The staining characteristics of this substance were very similar to those of the Reichert's membrane in parietal yolk sac (Pierce *et al.*, 1963). Moreover, structures resembling normal parietal and visceral yolk sac could be observed in the tumors (Sobis and Vandeputte, 1973). Metastatic tumors showed a histological picture very similar to that of the primary neoplasm. Groups of neoplastic cells were frequently found in the blood vessels. In the ascites fluid, structures similar to embryoid bodies described in mouse teratocarcinoma (Pierce *et al.*, 1960) were seen. They were composed of endodermal cells which surrounded the core of hyalin; we call them embryoid-like bodies (Fig. 4).

The ultrastructure of the tumors was very similar to the ultrastructure of yolk sac carcinoma isolated from a mouse testicular teratoma (Stevens and Hummel 1957; Pierce *et al.*, 1962; Pierce and Beals, 1964) and to that of yolk sac carcinomas derived from the extraembryonic part of mouse egg cylinders transplanted under the kidney capsule (Solter and Damjanov, 1973). In all tumors a grey substance separating individual or groups of neoplastic cells was observed. This substance was also present in dilatated rough-surfaced endoplasmic reticulum of numerous tumor cells. Other cells were less differentiated with many free ribosomes and normal mitochondria. The cell membranes were regular, desmosomes were observed. In most of the tumors C-like particles were present in a few cells. In the transplanted neoplasms virus particles were no longer observed.

These tumors, diagnosed as yolk sac carcinomas, were easily transplantable in syngeneic rats. In the sera of the rats bearing either a primary or a transplantable yolk sac carcinoma, increased levels of alphafetoprotein (AFP) were detected (Hooghe *et al.*, 1974). By immunofluorescence it was determined that the AFP was secreted by the yolk sac carcinoma cells displaying the visceral type (Delacourt *et al.*, 1976).

Several *in vitro* cell lines could be established from primary or transplanted tumors. The cells formed a rather homogeneous layer of epithelial-like cells with PAS-positive, diastase-resistant vacuoles. Isolated masses of cells often detached from the plastic and floated freely in the medium. On section they consisted of a central core of PAS-positive, diastase-resistant material surrounded by several layers of endodermal-like cells.

Using the same method (fetectomy and MSV inoculation) we also obtained a few tumors consisting of poorly differentiated cells (Fig. 5) and of areas containing well differentiated tissues. The fetal origin as well as the histological characteristics of these tumors suggested that they may belong to the group teratocarcinoma. The

Fig. 2. Yolk sac carcinoma composed of visceral and parietal pattern. *H and E. x80.*

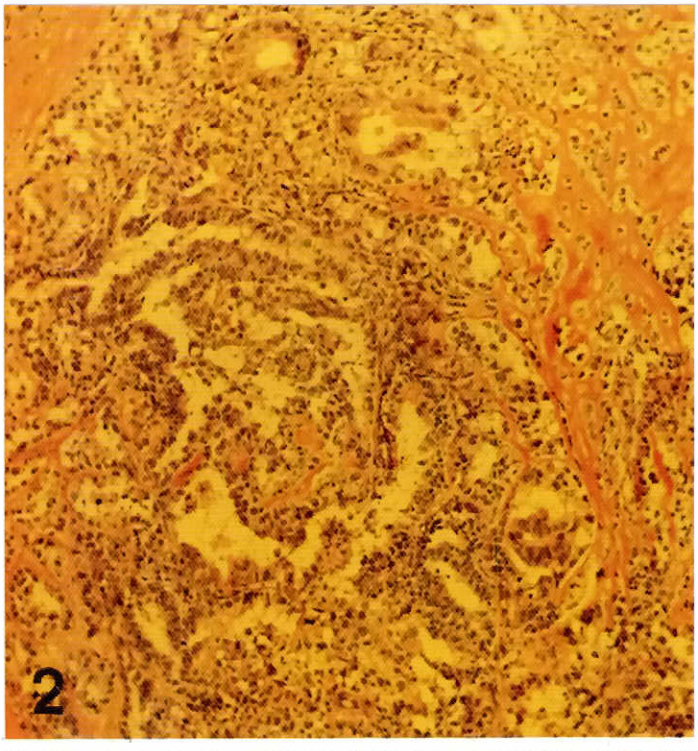
Fig. 3. PAS-positive substance in the cytoplasm and between the cells. *PAS. x830.*

Fig. 4. Embryoid-like body from ascites. *H and E. x360.*

Fig. 5. Embryonal carcinoma. *H and E. x360.*

Fig. 6. Small focus of yolk sac carcinoma 9 days after MSV inoculation. *PAS. x35.*

Fig. 7. Trophoblastic cells in yolk sac carcinoma. *H and E. x80.*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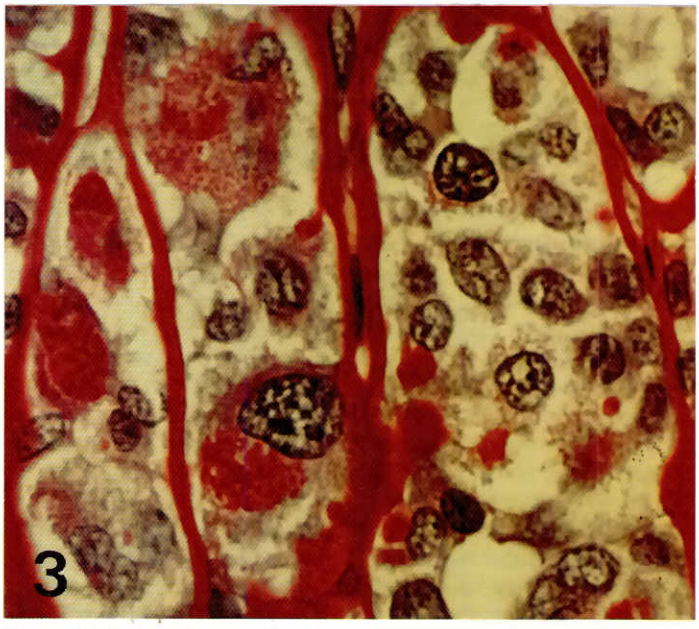
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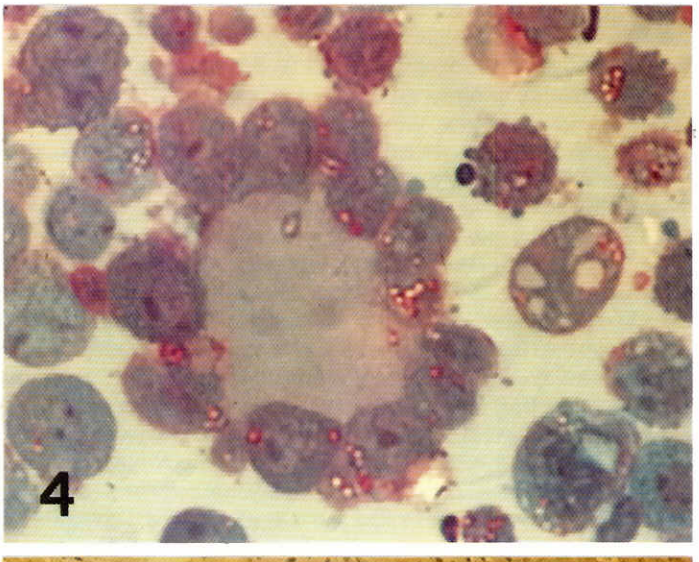
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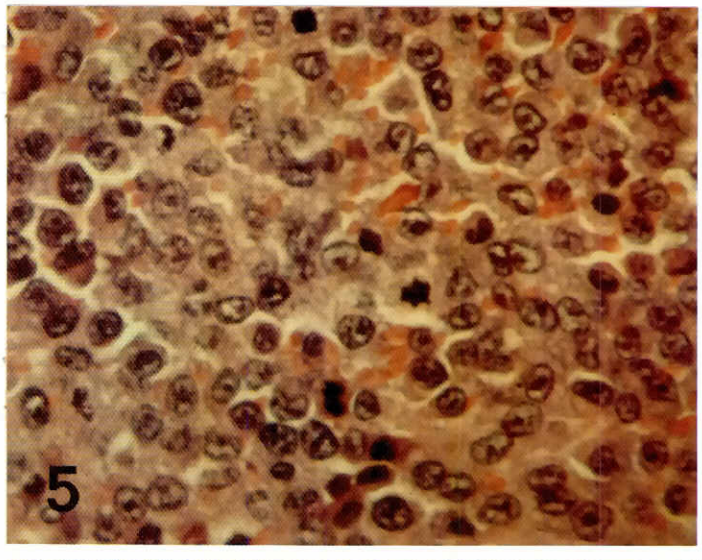
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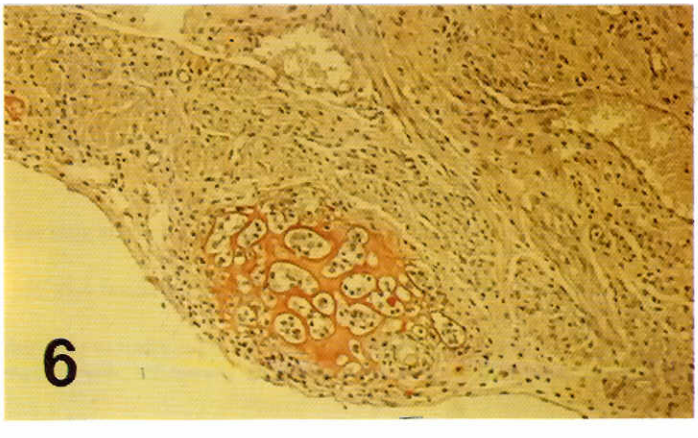
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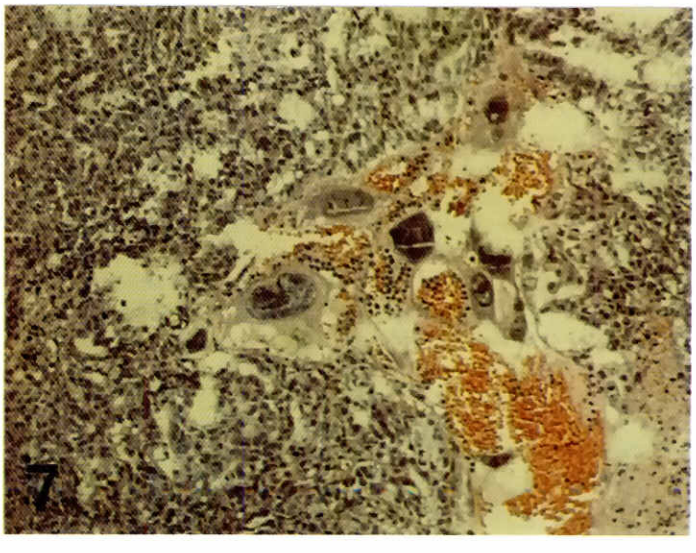
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poorly differentiated cells grew very well *in vitro* in tissue culture and *in vivo* after transplantation into syngeneic hosts. The morphology of these cells and the presence of high alkaline phosphatase activity qualifies them as embryonal carcinoma cells (Sobis and Vandeputte, 1976a). Moreover, these cells have a potential to differentiate mostly into yolk sac carcinoma and occasionally also in other tissues.

Some years after our observation that MSV could induce extraembryonal tumors when inoculated in the placenta of fetectomized rats, it was shown by Sakashita *et al.* (1977) and ourselves (Vandeputte and Sobis, 1978) that displacement of the visceral yolk sac outside the uterus of fetectomized animals without MSV inoculation also led to the development of yolk sac carcinomas in rat and hamsters (Sobis and Vandeputte, 1977). Like the MSV-induced yolk sac carcinomas these tumors metastasized and killed the host. They are transplantable in syngeneic rats, grow in tissue culture *in vitro* and are not immunogenic.

This observation let to the study of the role played by the MSV in the tumor induction as initially described. By comparing the biological, morphological and immunological characteristics of the neoplasms which develop in animals injected or not with the virus, we found that MSV inoculation into the placenta of fetectomized rats does indeed play a definitive role in the induction of visceral yolk sac-derived malignant tumors (Van Hove *et al.*, 1982). This conclusion was based upon the following findings: 1) the tumor incidence was significantly greater in the MSV-inoculated group; 2) the latency period was markedly shortened (average 10 weeks versus 23 weeks for the non-virus group); 3) all the early tumors (latency period of less than 3 months) in the virus group appeared in the MSV-inoculated uterine horn; 4) in rats preimmunized with MSV the results were quite similar to those recorded in animals treated with fetectomy alone and 5) only in the virus group did we observe the development of less differentiated tumors (embryonal carcinoma).

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. These cells are likely to be rejected by a process of immune selection to yield a population of non-producer cells as is found in the yolk sac carcinomas. In order to confirm this hypothesis and to verify whether the shorter latency period in the viral-induced yolk sac carcinomas 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 placentas. The results of this study indicate that MSV facilitates the malignant transformation of visceral yolk sac cells (Vandeputte and Sobis, 1988). In spite of the histological similarity of the proliferating but still benign cells in the displaced visceral yolk sac in both groups of rats killed at days 3 and 6 after fetectomy, at the ultrastructural level C type particles were found only in MSV-inoculated animals. These particles were no longer observed at the later stages. As early as 9 days after virus inoculation foci of tumor cells were observed (Fig. 6). The number and volume of these malignant foci increased gradually afterwards.

In contrast, no morphological signs of malignant transformation were recorded during the same time period in the displaced membrane of animals 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 yolk sac carcinomas by acting as a transforming agent on the yolk sac cells or by another mechanism, e.g. by influencing the host immune response. To approach this problem we looked for the presence and expression of *mos* sequences in spontaneous and in Mo-MSV-induced rat yolk sac carcinomas. While C-*mos* was found in all tumors tested, only in the viral-induced neoplasms were additional *mos*-sequences recorded. The latter were identified as randomly integrated Mo-MSV provirus (Decock *et al.*, 1987). In none of the yolk sac carcinomas induced by displacement alone (spontaneous) were these v-*mos* sequences observed. This indicates 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 in any of yolk sac carcinoma-derived cell lines.

Although no virus production was detected in the viral-induced yolk sac carcinomas, it is likely that at the initial stages of tumor outgrowth, virus producing cells are present since C-type particles were regularly observed at the early stages of cell proliferation (Vandeputte and Sobis, 1988). 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 of rat MSV-induced embryonal carcinoma cells into a producer line by superinfection with an endogenous C-type mouse virus, these cells no longer grew in syngeneic rats (Sobis *et al.*, 1980). 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 virus-producer cells an elevated level of v-*mos* RNA was found (Decock *et al.*, 1987).

Although the role played by the virus in the pathogenesis of the tumors is still not clear, we think that MSV transforms some stem cells present in the visceral yolk sac which proliferate in the displaced visceral yolk sac. When transformed, these stem cells may keep their undifferentiated character (pure embryonal carcinoma) or differentiate into yolk sac carcinoma. If no malignant transformation occurs, they only give rise to benign differentiated tissues (teratoma, cfr. infra). Since these stem cells can develop into all kinds of tissues, they have to be multipotential. Such multipotentiality is displayed by germ cells and early embryonal cells (until day 7). As the latter are not present in the yolk sac of 12-day-old embryos, we

Fig. 8. Gut-like structure in the teratoma. *H and E. x80.*

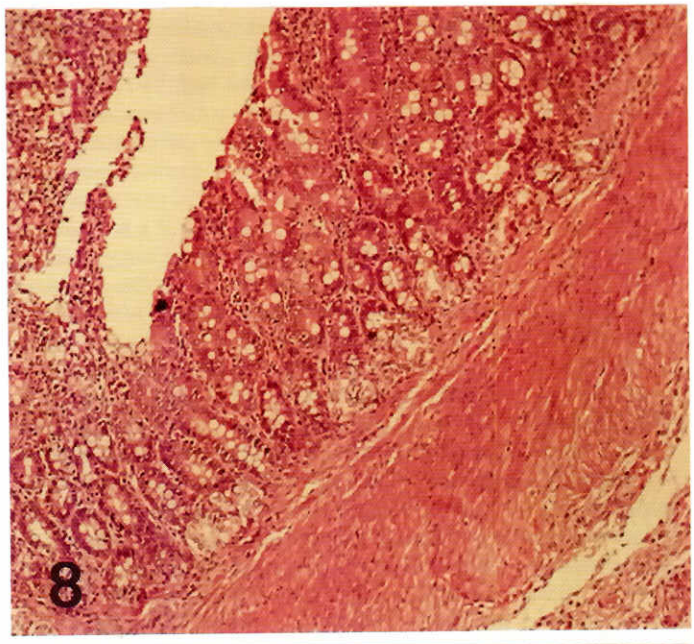
Fig. 9. Skin with its appendages and cartilage in the teratoma. *H and E. x35.*

Fig. 10. Nervous tissues in the teratoma. *H and E. x80.*

Fig. 11. Poorly differentiated cells in the displaced visceral yolk sac *in vivo*. *H and E. x360.*

Fig. 12. Poorly differentiated cells in the cultured visceral yolk sac *in vitro*. *H and E. x360.*

Fig. 13. Visceral yolk sac fixed 10 days after incubation *in vitro* with [^{3}H]thymidine. *Silver grains are present in the nuclei of endodermal mesenchymal and trophoblast giant cells. x830.*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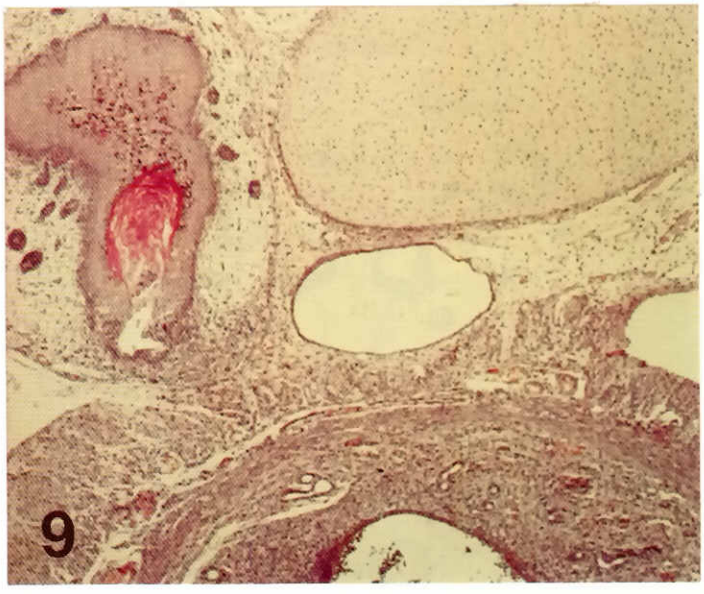
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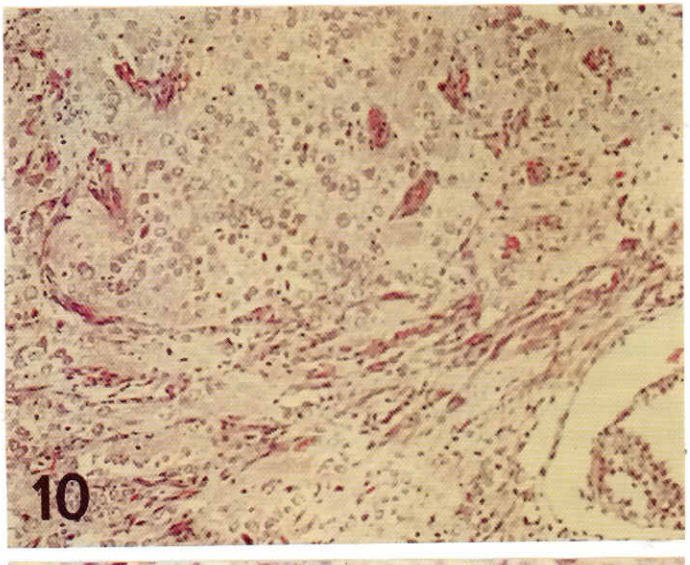
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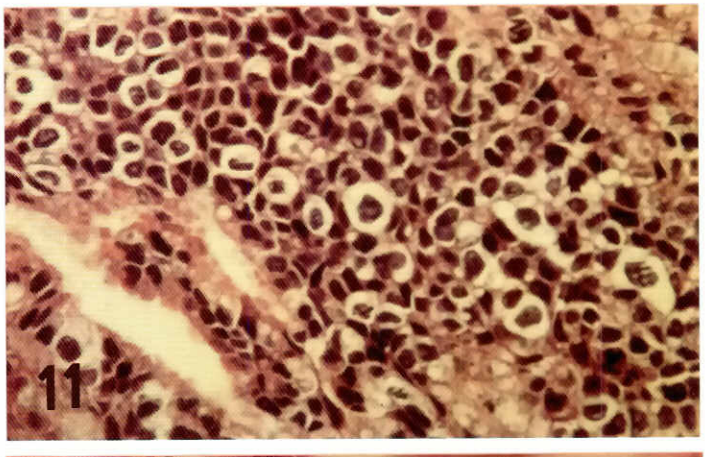
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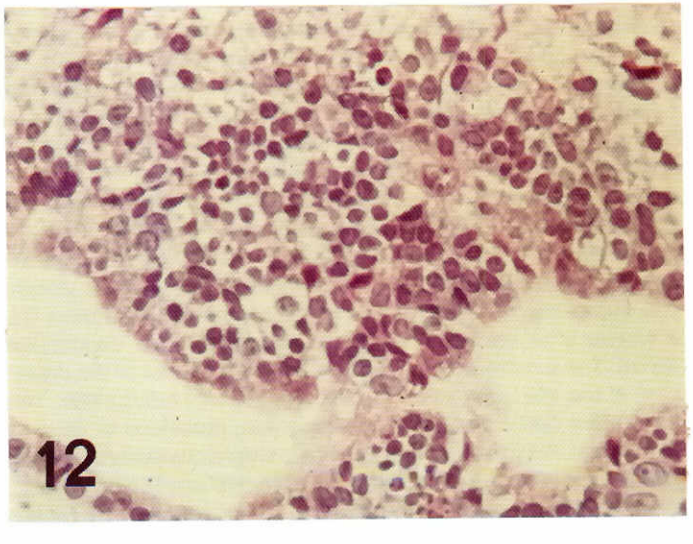
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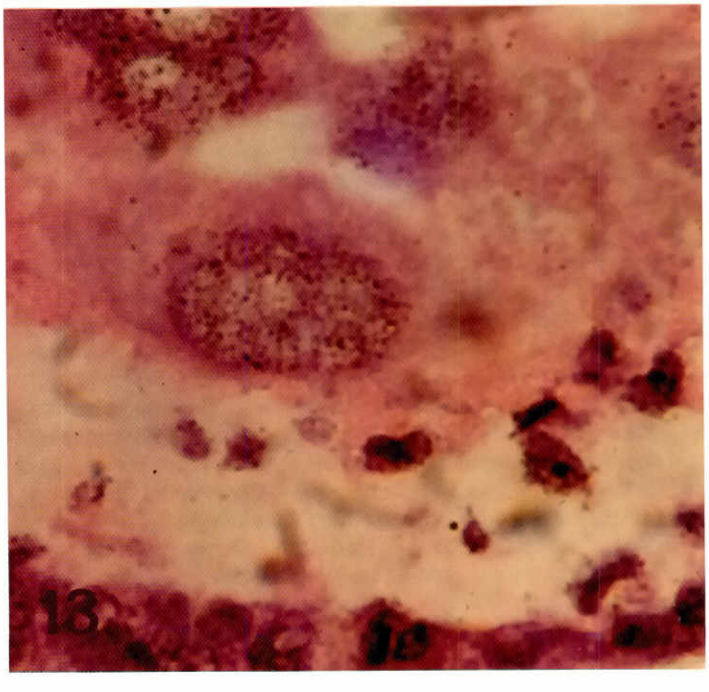
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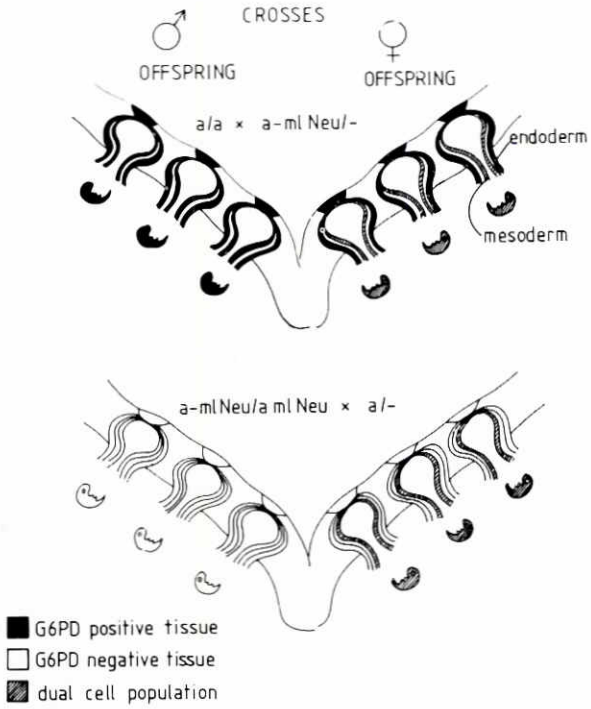


Fig. 14. Expected G6PD activity in fetuses and fetal membranes of fetectomized wild-type or homozygous enzyme-deficient females mated with hemizygous G6PD deficient or wild-type males.

verified whether the germ cells are not the stem cells of the yolk sac tumors. For this purpose we treated pregnant rats with Busulphan. This drug, when administered in appropriate doses, selectively destroys the primary germ cells during their migratory phase, mostly at day 10 of gestation (Gaulier and Roux, 1970; Hemsworth and Jackson, 1963). Indeed, we did not find any germ cells in the gonads of neonates from Busulphan-treated mothers (Sobis and Vandeputte, 1976b). Yet, this treatment did not influence the incidence or the latency period of malignant tumors induced in fetectomized rats, injected with MSV or not.

Moreover, we observed in 129 Sv/steel mice that the development of yolk sac carcinomas 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 (Sobis *et al.*, 1983). The results of these experiments prove that the yolk sac carcinomas are not of germ cell origin. Therefore, one has to postulate the appearance of multipotential cells others than germ cells in the displaced visceral yolk sac which give rise to different structures. Indeed, in the yolk sac-derived yolk sac carcinomas, besides cells displaying the parietal or visceral pattern, trophoblastic cells (Fig. 7) and mesenchymal structures were found (Sobis *et al.*, 1982a). It was also shown that cell clones of rat yolk sac carcinoma isolated *in vitro* may differentiate into parietal and visceral endodermal cells, into trophoblast and into mesenchymal tissue after reinoculation in the rat (Van Hove *et al.*, 1985a). This observation applies, however, only to visceral yolk sac-derived tumors. Indeed, in yolk sac carcinomas induced by transplantation of the extraembryonic part of the whole egg cylinder under the kidney capsule of a syngeneic host (Damjanov and Sell, 1977; Damjanov *et al.*, 1977), only visceral and parietal structures were found (Damjanov, 1980; Sobis *et al.*, 1982b). The differences in morphology between both types of yolk sac carcinomas can be explained by a difference in the developmental stage of the tissue giving rise to yolk sac carcinoma. The extraembryonic part of a 9-day-old egg cylinder contains young endoderm that proliferates to form biphasic endodermal yolk sac carcinoma. In contrast, the 12-day-old visceral yolk sac is composed of endoderm and mesoderm which are well differentiated. However, after displacement of this fetal membrane proliferation of the endodermal and/or mesodermal cells is accompanied by the appearance of poorly differentiated cells (Sobis and Vandeputte 1975; Vandeputte *et al.*, 1979; Sobis *et al.*, 1982b). The latter may be the stem cells of the yolk sac carcinoma. As previously suggested (Sobis *et al.*, 1982a) the mesenchymal tissue in this tumor may induce trophoblast differentiation. This inductive property of mesodermal tissue on the differentiation of trophoblast has been described during embryogenesis (Snell and Stevens, 1966; Gardner *et al.*, 1973; Peel and Bulmer 1977). In the embryo-derived yolk sac carcinoma no mesenchyme was found (Damjanov, 1980; Sobis *et al.*, 1982a). 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 yolk sac carcinoma. This hypothesis is further strengthened by our study of visceral yolk sac in organ culture (Lu *et al.*, 1984). Under appropriate conditions the proliferation of poorly differentiated cells can be observed together with the appearance of trophoblast cells secreting progesterone in close connection with the mesenchyme.

All these data support the suggestion of Gaillard (1973) that all extraembryonic tissues have a common origin. The observation on the mouse teratocarcinoma was restricted, however, to parietal endoderm and trophoblast (Gaillard, 1981; Damjanov *et al.*, 1985) while the cloning of visceral yolk sac-derived rat yolk sac carcinoma demonstrated the potentiality of a single cell to differentiate into visceral and parietal endoderm, mesoderm and trophoblast.

Teratoma

As mentioned before, we very frequently observed the development of benign tumors attached to the uterine horn in the fetectomized rats inoculated or not with MSV and independently from the presence of the malignant yolk sac carcinoma or embryonal carcinoma. These benign tumors were composed of well differentiated tissues. Since it has been described that the visceral yolk sac implanted into the omentum can differentiate into a few different tissues (Payne and Payne, 1961; Avery and Hunt, 1968), we verified whether this fetal membrane left outside the uterus after fetectomy was responsible for the development of these well differentiated tissues. We could indeed show that externalization of the visceral yolk sac in fetectomized rats led to the appearance of tumors 3 to 4 weeks after operation in all animals (Sobis and Vandeputte, 1974). The tumors were encapsulated and attached to the uterine horn at the site, where the visceral yolk sac was left outside. They were often cystic and contained a serous, mucinous or caseous substance. Histologically the neoplasms were characterized by the presence of a variety of well differentiated tissues. The endodermal cysts contained mucin and were lined with a columnar epithelium

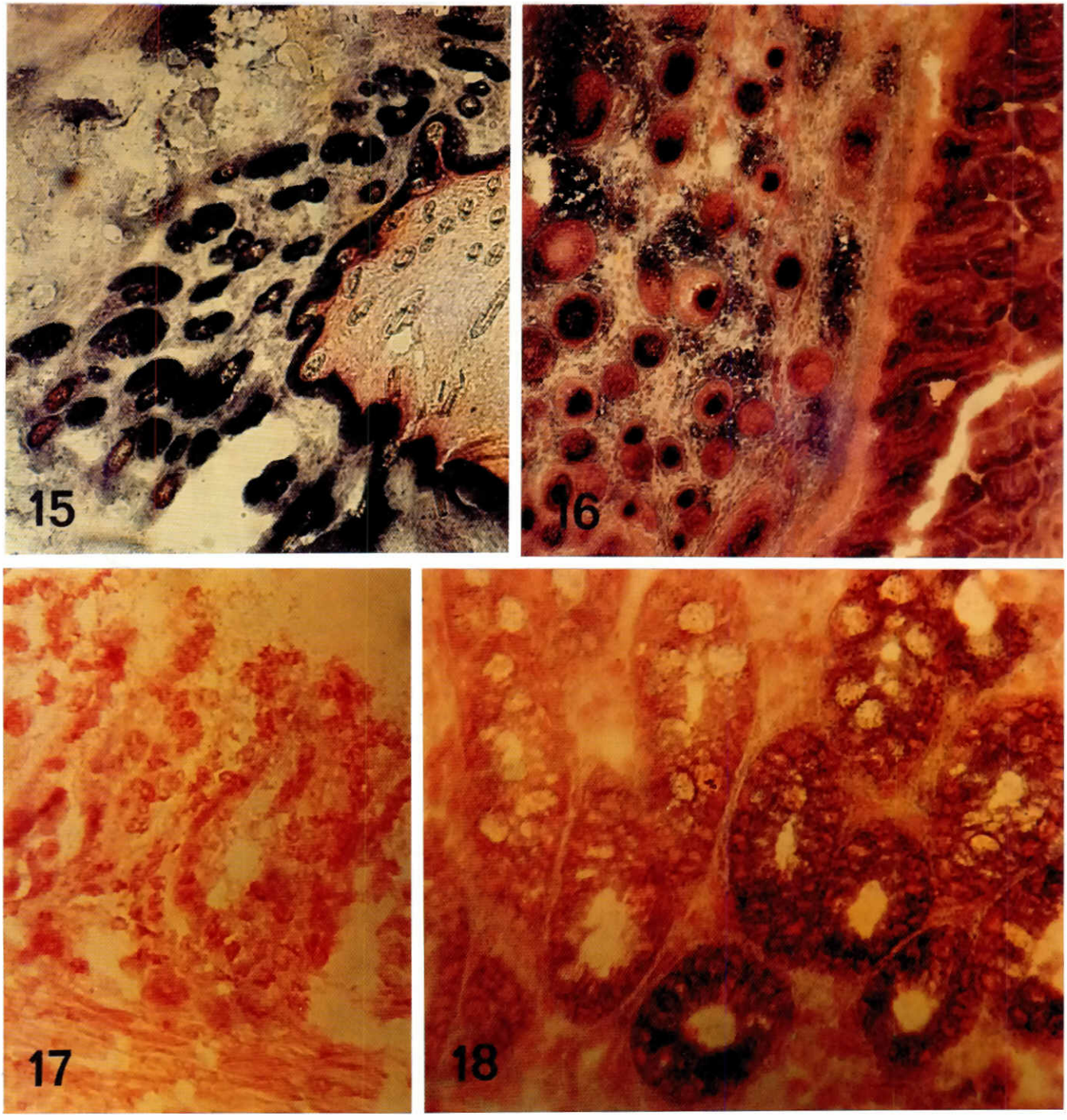
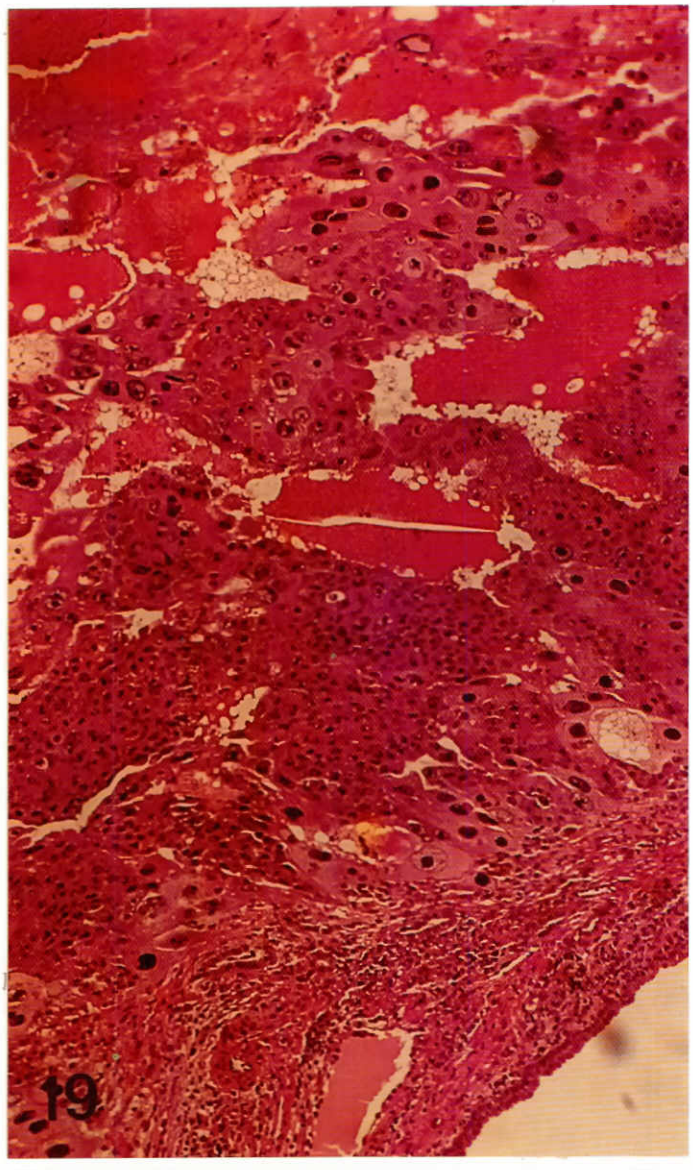


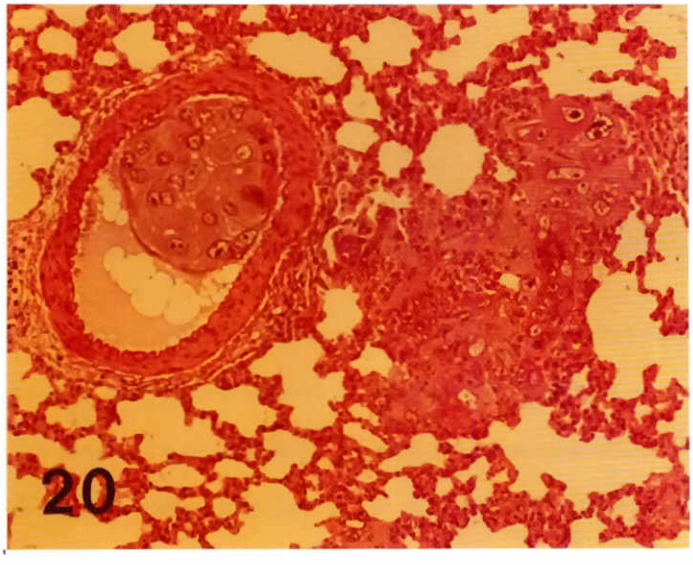
Fig. 15. Yolk sac-derived teratoma from wild-type female mated with hemizygous enzyme-deficient male. *All epithelial cells of skin and its appendages are G6PD-positive. x80.*

Fig. 16. Embryoma developed from heterozygous embryo from the same cross as teratoma shown in Fig. 15. *Dual G6PD-positive and -negative cells are seen in epithelium and skin appendages. x80.*

Fig. 17. Yolk sac-derived teratoma from homozygous G6PD-deficient female mated with wild-type male. *Gut-like structure is enzyme-negative. x360.*

Fig. 18. Embryoma developed from the same conceptus as the teratoma shown in Fig. 17. *In gut-like structure dual G6PD-positive and -negative cell population is seen. x360.*





with goblet cells. Their ultrastructure was very similar to that of the cells found in the gut. This endodermal epithelium regularly formed folds similar to intestinal villi with a central core of connective tissue. Smooth muscle oriented in transverse and longitudinal layers was regularly seen around the endodermal cysts (Fig. 8). Very often cartilage and bone with areas of endochondrial ossification were found. Bone marrow was present in the medulla of such bone and contained different cell types like erythroblasts, myeloblasts, megakaryocytes, plasma cells and reticulum cells. We frequently observed epidermal cysts containing keratin and lined by well differentiated squamous epithelium consisting of all the structural layers commonly present in mature skin. In the subadjacent connective tissue layer, skin appendages such as hair follicles and sebaceous glands were often present (Fig. 9). Ultrastructural examination revealed a high degree of differentiation in these skin derivatives. In about one third of the tumors we observed smooth and skeletal muscle and nervous tissue (Fig. 10). The striated muscular tissue was well differentiated with clearly defined sarcomeres and myofibrils. In the nervous tissue, neuroglial cells as well as neurons were observed and occasionally well differentiated ganglionic neurons and meningeal formation. Less frequently the tumors contained bronchiolar epithelium, thyroid, salivary glands, gastric epithelium and thymus. All these morphological features are characteristics for benign teratomas.

We never succeeded in obtaining outgrowth after transplantation into syngeneic host or to establish *in vitro* continuous cell lines from these tumors.

These visceral yolk sac-derived teratomas show some similarity with experimental teratomas induced by Nicholas (1942) by extrauterine implantation of early egg or egg cylinder and embryonic parts of $7^{1/2}$ and 9-day-old rat embryos (Skreb *et al.*, 1971). In these experiments the tumors were derived from embryonal or primordial germ cells and not from fetal membranes. In our model, in order to induce teratomas containing all described tissues, the fetectomy with externalization of the visceral yolk sac has to be done not later than day 12 of pregnancy (Sobis and Vandeputte, 1979). Fetectomy and displacement of the visceral yolk sac at later stages of development, e.g. day 13 to 15, gave a much smaller variety of differentiating tissues.

With the same technique one can induce similar teratomas in mouse and hamster. Here too the age of pregnancy at the time of operation is crucial. In the mouse day 11 of pregnancy is ideal, in the hamster day 9.

The teratomas could only be obtained in pregnant animals whose fetuses were histocompatible. The semi-allogeneic visceral yolk sac expressing paternal antigen was rejected when placed outside the uterus (Sobis *et al.*, 1978). Only after induction of active or passive immunological enhancement could the hybrid visceral yolk sac develop into benign teratomas. These hybrid teratomas contained, however, fewer mesodermal derivatives (bone, bone-marrow and muscle) than the syngeneic ones. Cartilage, ectodermal and endodermal tissue were nearly as frequent in the hybrid as in syngeneic teratomas.

Fig. 19. Choriocarcinoma composed of cytotrophoblasts and giant cells. *H and E. x80.*

Fig. 20. Choriocarcinoma cells in the blood vessels of the lung. H and E. *x80.*

Gaillard (1974) distinguishes 3 steps of differentiation in teratomas: immature differentiation, embryonic differentiation and organogenesis. Since in the experiments reported we detected only the mature differentiation and organogenesis, we performed sequential morphological studies in order to monitor the appearance and the differentiation of all the tissues formed in these tumors (Sobis and Vandeputte, 1975). We found that the endoderm and mesoderm of the visceral yolk sac left outside the uterus after fetectomy starts already to proliferate 2 days after operation. This proliferation was observed in an increasing number of cases during the following 3 days and diminished afterwards. Apart from these proliferating endodermal and mesodermal cells, poorly differentiated cells also appeared in the displaced yolk sac (Fig. 11). These cells show neither mesodermal nor endodermal characteristics. They do not express endodermal antigen (Vandeputte *et al.*, 1979). The poorly differentiated cells were present in the displaced visceral yolk sac until day 9, but were most numerous at days 4 and 5 (Sobis *et al.*, 1982b). The organoid structures (like gut, pancreas, cartilage) started to appear at day 8 after operation, and at day 19 all elements found in mature teratomas were already present (Sobis and Vandeputte, 1975). Since in these tumors the derivatives of 3 germ layers were observed and since the visceral yolk sac is composed only of endo- and mesoderm, one has to consider the possibility that multipotential cells are responsible for the development of the teratomas. Cells considered as multipotential and from which derivatives of all 3 germ layers can originate are germ cells or early embryonal cells (Stevens, 1967; Minz *et al.*, 1978). A germ cell origin for the yolk sac-derived teratomas is not likely for several reasons: 1) the part of the membrane pulled through the uterine wall is not the one near the allantois in which the primary germ cells are found at day 9 (Chiquoine 1954). Moreover, Ozdzenski (1969) has shown that the primordial germ cells do not originate in the yolk sac, but instead in the roof of the allantois and the hind region of the embryo. Furthermore, these cells, the structure of which is very characteristic (Spiegelman and Bennett, 1973; Zamboni and Merchant, 1973; Eddy, 1974) were never found in serial 1 µm sections made of 12-day-old visceral yolk sac (Sobis and Vandeputte, 1975). 2) Treatment with busulphan, which is known to destroy germ cells during their migratory phase, did not influence the number or the morphology of the yolk sac-derived teratomas (Sobis and Vandeputte, 1976b). 3) Using the appropriate crosses between Steel-Sl/+ females and Sl/+ males we demonstrated that the lack of germ-free cells does not inhibit the development of yolk sac-derived teratomas in these mice (Sobis and Vandeputte, 1982). The displaced yolk sacs belonging to genetically sterile embryos developed into teratomas as frequently as those from heterozygotes and from genetically normal embryos. 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. Such multipotential cells may be initially present in the visceral yolk sac or they may arise by dedifferentiation. Since we never observed poorly differentiated cells on serial sections made through many rat yolk sacs examined at different stages of their development during normal pregnancy, we rather favor the hypothesis that they arise by a process of dedifferentiation after displacement of the yolk sac. We did indeed observe these poorly differentiated cells in the displaced yolk sac *in vivo* as well as *in vitro* (Fig. 12). Under special organ culture conditions, poorly differentiated cells do indeed proliferate in the explanted visceral yolk sac and differentiate into endodermal and epidermal cysts and trophoblast giant cells (Fig. 13) (Lu *et al.*, 1984).

Since the visceral yolk sac is composed only of mesoderm and endoderm (but not ectoderm), the dedifferentiated cells and the various well differentiated tissues derived from them have to originate from one of these two layers. To verify the origin of these proliferating and differentiating cells we labeled the visceral yolk sac *in vitro* with [^{3}H]thymidine and performed autoradiographic studies after *in vitro* culture and after transplantation *in vivo* (Sobis *et al.*, 1986). The results of these experiment showed that both endodermal and mesodermal cells of the 12-day-old visceral yolk sac incorporate [^{3}H]thymidine in their nuclei. Three days later the label was found in proliferating poorly differentiated cells and afterwards in differentiated tissues (Fig. 13) (e.g. squamous epidermis, endodermal cysts and giant trophoblast cells). Whether these tissues originate from the endoderm or from the mesoderm could, however, not be determined. To further verify the origin of poorly differentiated cells we looked for the kind of intermediate filaments present in these cells and their ultrastructural relation to the basement membrane. We found that the poorly differentiated cells contain keratin filaments. On the contrary, antivimentin antibodies reacted only with the mesodermal tissues (Sobis *et al.*, 1989). Moreover, the poorly differentiated cells were present at the same side of the basement membrane as the endodermal cells. Since these results favor the hypothesis of endodermal origin of poorly differentiated cells, we tried to find features of retrodifferentiation of the endodermal cells. For this, we chose plant lectins which react with the rat endoderm of visceral yolk sac at different stages of their maturation (Sobis and Vandeputte, 1989). Using four different lectins reacting with young endoderm we obtained a strong reaction with both the proliferating endoderm and the poorly differentiated cells. This might indicate that these proliferating endodermal cells which are still positive for yolk sac antigen 1 (cf. below) retrodifferentiate and thereby acquire the receptors for lectins which are typical for earlier visceral endoderm.

Although the results of all these experiments indicate that the yolk sac-derived teratomas are most probably of endodermal origin, definitive proof was still lacking. Such proof could only be provided by a marker that distinguishes endodermal from mesodermal derivatives during embryogenesis and fetal development. An X-linked enzyme marker would be very helpful because in the female embryo one of the X-chromosomes is inactivated (Lyon 1961, 1972). This inactivation is random in embryonal ectoderm but the paternal X-chromosome is preferentially inactivated in the trophectoderm and in the yolk sac endoderm (Takagi and Sasaki, 1975; Wake *et al.*, 1976; Frels and Chapman, 1980; Harper *et al.*, 1982; West, 1982).

Therefore, we used mutant mice deficient in X-linked glucose-6-phosphate dehydrogenase (G6PD) (Pretsch *et al.*, 1988) to induce teratomas and embryomas. The presence of this enzyme can be revealed by histochemical method (Thomas *et al.*, 1988). The differences in the enzyme activity between yolk sac mesoderm and embryo versus yolk sac endoderm and trophoblast can be detected in female concepti by using appropriate crosses of wild-type and G6PD-deficient mice (Fig. 14) (Sobis *et al.*, 1991a). We compared histochemically the G6PD activity in the tissues differentiated from the visceral yolk sac pulled outside the uterus with the tissues developed from corresponding embryos transplanted under the kidney capsule (embryomas). The results clearly showed that nearly half of the embryomas and all teratomas from crosses of wild-type

females with hemizygous enzyme-deficient males contained tissues uniformly positive for G6PD (Fig. 15). The other half of embryomas was composed of tissues containing a dual (G6PD + and -) cell population (Fig. 16). The corresponding teratomas were G6PD-positive. In the second combination composed of homozygous G6PD-deficient females and wild type males, all teratomas and half of the embryomas were nearly devoid of the enzyme (Fig. 17). The other half of the embryomas were composed of dual cell population (Fig. 18). These results clearly indicate that the teratomas develop from a tissue in which the parental X chromosome is inactivated and therefore originate from the endoderm of the visceral yolk sac (Sobis *et al.*, 1991b). Moreover, the data obtained exclude a germ cell origin of the yolk sac-derived tumors. Indeed, it is known that the germ cells develop from ectoderm in which inactivation of one of the X chromosome is random (McMahon *et al.*, 1981; Gardner *et al.*, 1985).

Since the results of all cited studies prove that the well differentiated endodermal cells of visceral yolk sac dedifferentiate, proliferate and redifferentiate into derivatives of all 3 germ layers, this phenomenon has to be considered as transdifferentiation (Eguchi, 1976). By which mechanism the mature yolk sac endodermal cells can switch to form ectodermal and mesodermal organoid structures has still to be determined.

Choriocarcinoma

As stated previously, the poorly differentiated cells in the displaced visceral yolk sac can also differentiate in trophoblast *in vivo* as well as *in vitro*. Trophoblast is a remarkable tissue in that it shares several characteristics with neoplastic cells and also plays an important role as quarantining layer to protect the fetus against immune aggression of the mother. Unfortunately, trophoblast cells are difficult to isolate in pure form and do not grow *in vitro* for a sufficiently long time as to allow certain experiments. An alternative model is choriocarcinoma, which although malignant, displays most characteristics of normal trophoblast. Such a choriocarcinoma was kindly provided to us by Teshima. The tumor had been induced by Teshima *et al.* (1983) in WKA/H rats with our technique of displacement of yolk sac in fetectomized rodents. It is to our knowledge the only rat choriocarcinoma available as transplantable tumor. Whether transplanted subcutaneously or grafted under the kidney capsule, the tumor looks very hemorrhagic. Its growth did not depend upon the sex or the strain of rats used. Even after preimmunization the grafted tumor cells grew as well in the allogeneic (R/A and BN rats) as in the syngeneic (WKA/H) host (Verstuyf *et al.*, 1989).

Histologically the neoplasm is composed of giant cells surrounding spaces filled with blood and proliferating cytotrophoblast (Fig. 19). Groups of choriocarcinoma cells were often found in blood vessels. Small metastases were observed in the lungs (Fig. 20), rarely in lymph nodes. The tumor secretes placental lactogen as demonstrated by proliferation and secretion of the mammary glands of tumor-bearing animals, independently of their age or sex. By histochemistry the presence of Δ^5-3ß-hydroxysteroid dehydrogenase was shown in the giant cells. The tumor was further shown to express placental lactogen type I mRNA and protein but no type II prolactin-like protein A (PLP-A) nor prolactin-like protein B (PLB-B) (Faria *et al.*, 1990). These properties indicate that the hormone secretion by the tumor cells is very alike to that of normal placental cells. Under electron microscopy, three types of cells were distinguished: 1) poorly differentiated cytotrophoblast cells; 2) giant cells which often formed three layers bordering the spaces filled with blood cells similar to those described in labyrinth placenta of the rat (Metz *et al.*, 1976; Metz, 1980) and 3) a second type of giant cells, larger and comparable to the giant cells of the junctional zone of normal rat placenta (Jollie, 1981).

From this transplantable rat choriocarcinoma we succeeded to obtain an *in vitro* cell line (Verstuyf *et al.*, 1990). The cell culture is composed of pure trophoblast cells which multiply and differentiate. The morphology of the cells is very similar to normal rat cytotrophoblast and giant cells. The epithelial character of the cells is demonstrated by the presence of cytokeratin and receptors for the lectin Bandeira simplicifolia Agglutinin 1 (Wu *et al.*, 1983; Sobis and Vandeputte, 1989). The giant cells are hormonally active as demonstrated by the presence of lactogen and progesterone in the supernatant of the culture. To exclude the presence of endodermal cells of yolk sac origin in this cell line we used monoclonal antibodies directed against yolk sac antigen 1 and 2 (cf. below). No cells in the culture reacted with the antibodies. Chromosome analysis revealed a hyperdiploid male rat karyotype, which prove the paternal origin of the primary tumor developed from extraembryonic membranes.

The choriocarcinoma cell line only grows in a rich medium consisting of RPMI 1640 medium supplemented with glucose, sodium pyruvate and antibiotics, ß2 mercaptoethanol and fetal calf serum. Without these elements the cells do not multiply.

The influence of different growth factors on the growth of the choriocarcinoma cells was examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. It was found that only epidermal growth factor and to a certain extent insulin had a growth promoting effect by themselves. The other growth factors had either an additive effect in the presence of epidermal growth factor or no effect at all. The cytotrophoblast cells expressed both epidermal growth factor and transferrin receptors, whereas the more differentiated giant cells only expressed transferrin receptors (Verstuyf *et al.*, in press).

Since cytotrophoblast differentiate into giant cells which no longer proliferate and die, this tissue culture represents a good model to verify the action of drugs on cell differentiation. The advantage of the choriocarcinoma above other tumors for the study of differentiation is that 1) the differentiation stage of the cells is easily observed under the microscope; 2) the hormone secretion is dependent upon the cell type present and 3) the percentage of cytotrophoblastic cells can be determined by a specific monoclonal antibody recently obtained (cf. below). For instance, it was shown

Fig. 21. Endodermal antigen expressed on the apical pole of visceral yolk sac. *x360.*

Fig. 22. Endodermal antigen expressed on surface of proliferating visceral ectoderm. *x360.*

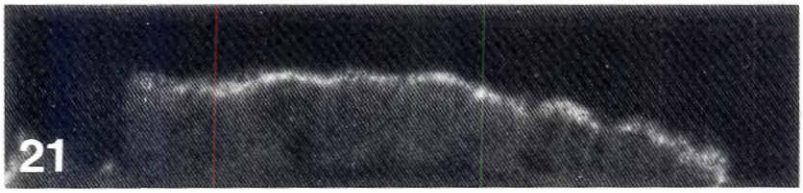
Fig. 23. Yolk sac antigen I expressed on visceral endoderm. *x360.*

Fig. 24. Yolk sac antigen II expressed on parietal endoderm. *x360.*

Fig. 25. Monoclonal antibody 22H6 reacts with cytotrophoblast of choriocarcinoma. *x360.*

Fig. 26. Monoclonal antibody reacts with hepatocytes. *x360.*

Fig. 27. Monoclonal antibody reacts with cytotrophoblast of junctional zone of rat placenta. *x360.*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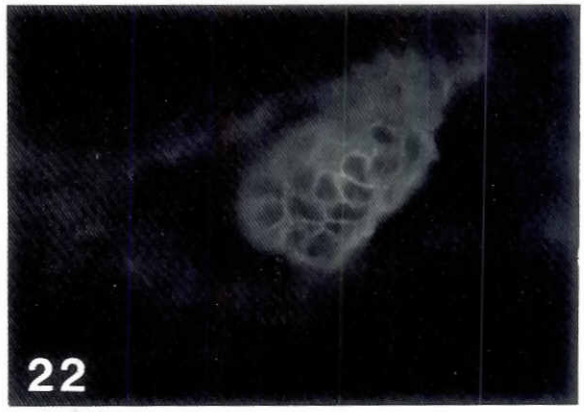
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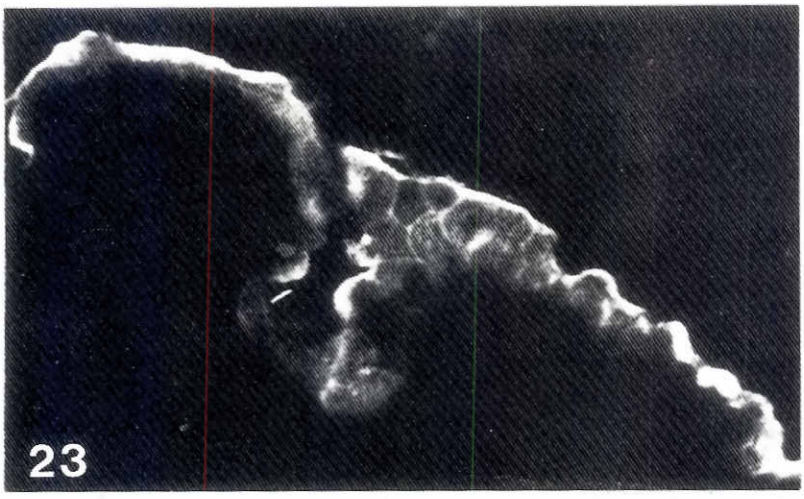
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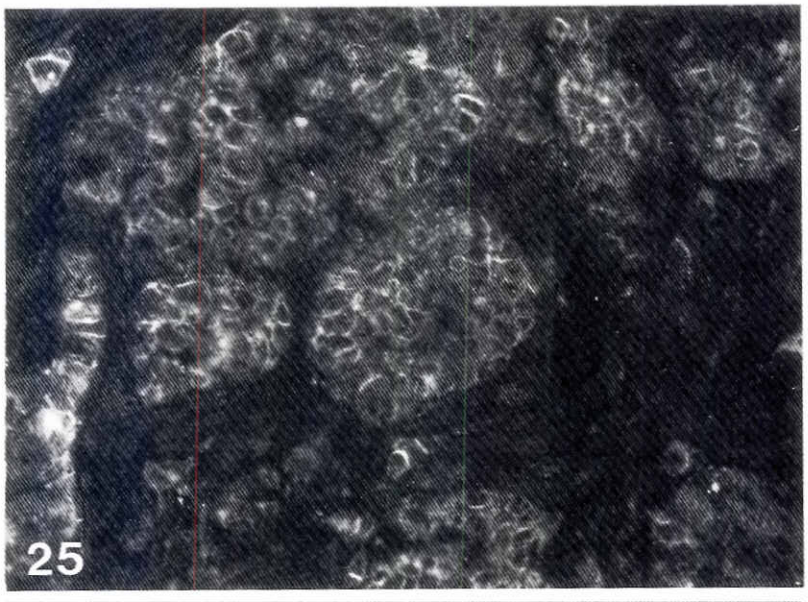
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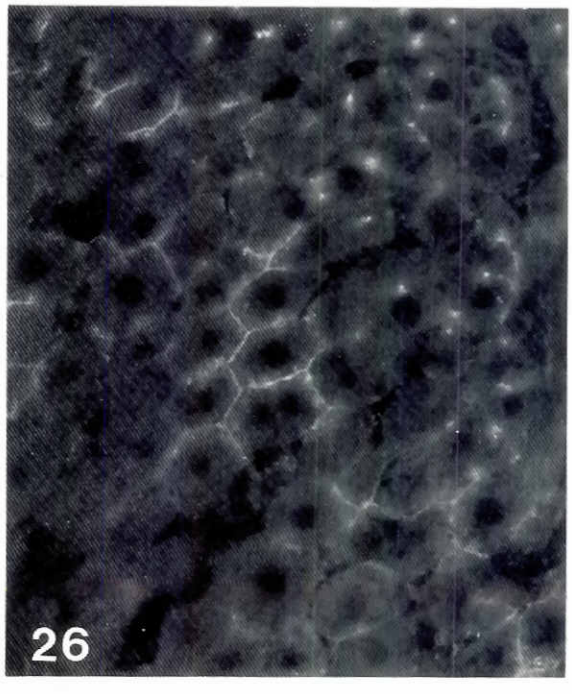
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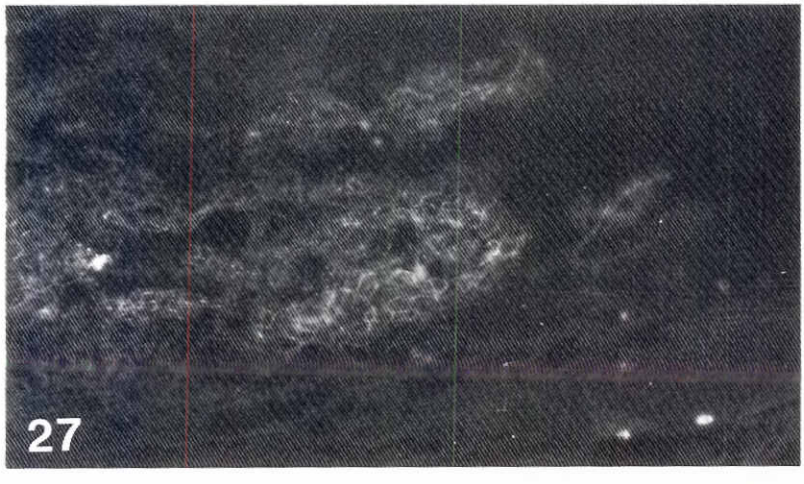
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that methotrexate does not kill the cytotrophoblast cells, but induces differentiation into giant cells *in vitro* (results not published).

Oncofetal antigens detected in extraembryonal fetal tumors

Many changes in the developmental state of a cell are accompanied by changes in the antigens of the cell surface. During embryogenesis, the cell surface antigens also play an important role in the development of the embryo. Immunological studies on these antigens, however, are made very difficult during these early stages of embryogenesis by the rapid changes in the differentiation pattern and hence in the associated differentiation surface antigens. Also the scarcity of embryonic material available and the inherent technical difficulties in obtaining one particular tissue without contamination by other cell types limits these studies. Fortunately some of these difficulties in the characterization of early embryonal differentiation antigens can be partly overcome by the use of stable lines of tumor origin. These cell lines may still possess the immunological characteristics of embryonal cells at particular stages of their development. Since these tumor lines are available in large quantities they can be used for the production of polyclonal or monoclonal antibodies that define embryonic antigens. Several cell lines derived from mouse teratocarcinoma, e.g. the F9 and PCC4, have been extensively used for this purpose and have made possible the characterization of several oncofetal and embryonal antigens (Solter and Knowles, 1978; Hamasima *et al.*, 1985).

Also, the extra-embryonic fetal tumors we described could be expected to be a good model for the study of both oncofetal and differentiation antigens. Indeed, both their fetal origin and their potentiality to differentiate in tissues belonging to different germ layers are likely to be linked to the expression of these antigens. The following experimental data confirm this assumption.

We first described the detection of an «endodermal» antigen using a rabbit anti-rat yolk sac carcinoma serum made monospecific by *in vitro* and *in vivo* adsorption. This antiserum was found to react with an antigen present on rat yolk sac carcinoma cells, on the endodermal cells of normal yolk sac of rat, mouse and hamster, on primitive endoderm of rat and mouse embryo and on yolk sac carcinoma and one adenocarcinoma of the rectum in man (Van Hove *et al.*, 1978; Sobis *et al.*, 1979). The antigen is spread over the cell surface of endodermal yolk sac cells until day 12 of pregnancy. Two days later it is concentrated at the apical pole of the more differentiated cells (Fig. 21). In the first stages of teratoma formation (cf. above), the proliferating endodermal cells of displaced visceral yolk sac express the antigen on the whole cell surface (Fig. 22) instead of at the apical pole as in the intrauterine yolk sac of that age. This may indicate that they revert to a lesser stage of differentiation (Vandeputte *et al.*, 1979).

Since this «endo-antigen» revealed by monospecific conventional antiserum may be of great interest as a marker for endodermal differentiation and for detection of certain tumors of endodermal origin, we decided to define it better by the technique of monoclonal antibodies (mab). We obtained several different clones producing mab which react with the yolk sac carcinoma. Two of them, the 6D1 mab and 3C3 mab, have been extensively examined. The 6D1 mab reacts with all rat yolk sac carcinoma cells displaying a visceral pattern but not with other rat tumors. It defines a stage-specific antigen for the normal visceral endoderm (Fig. 23) and embryonic endoderm (Van Hove *et al.*, 1984). The 6D1 is an IgG1 immunoglobulin which is not cytotoxic in the presence of complement. The only adult rat tissues reacting with this 6D1 mab are the spermatozoa and certain cells of the spermatogenic lineage. Further studies on rat spermatogenic cells indicated that the antigen defined by 6D1 mab was detected on the pachytene spermatocytes, spermatids and spermatozoa. Less differentiated cells of spermatogenesis like spermatogonia and early spermatocytes showed no reaction (Sobis *et al.*, 1988a).

The 3C3 mab detects a rat-specific antigen (yolk sac antigen 2) present on the normal parietal yolk sac cells (Fig. 24) and on the yolk sac carcinoma cells displaying a parietal yolk sac pattern. This rat yolk sac antigen 2 is also present at the apical pole of the epithelial cells lining the fetal and adult gut and kidney tubules (Van Hove *et al.*, 1985b). The 3C3, an IgG2a immunoglobulin is cytotoxic in the presence of complement.

The embryo-toxic effect of both mabs (6D1 and 3C3) has been verified in pregnant rats in order to investigate the biological role of these antigens defined by corresponding mabs. Our results indicate that after injection in pregnant rats: 1) both mabs can be shown to be localized specifically in the rat yolk sac (the 6D1 at the apical pole of the visceral endoderm and the 3C3 on the parietal yolk sac cells); 2) the 3C3 mab is embryotoxic (induces abortion) in a dose-dependent way whereas the 6D1 has no such effect; 3) both mabs are fixed on the corresponding structures of transplanted yolk sac carcinoma (the 6D1 on the visceral structure, the 3C3 on the parietal yolk sac) (Sobis *et al.*, 1988b).

To detect oncofetal antigens present on extraembryonal tissues like the placenta and more specifically on trophoblast, we immunized Balb/c mice with rat choriocarcinoma (cf. above) to prepare monoclonal antibodies. Between different clones we chose the 22H6 clone, which reacts only with cytotrophoblasts of rat choriocarcinoma (Fig. 25). The giant cells do not display a positive reaction. The antigen was shown on frozen sections and on tissue culture by indirect immunofluorescence (Verstuyf *et al.*, 1992). It is not expressed on tumors other than choriocarcinoma and is species specific. In adult rats the only cells revealing a positive reaction are hepatocytes (Fig. 26) and the epithelial cells lining the small intestine. In the pregnant rat, the antigen is expressed on the cytotrophoblasts of the junctional zone in the placenta (Fig. 27), but not on the giant cells. The mab reacts with the small trophoblast cells of the ectoplacental cone, but not with trophectoderm of blastocyst. The mab 22H6 has an IgG2b isotype and is not cytotoxic for choriocarcinoma cells in a complement-dependent cytotoxicity test.

This mab will make it possible to follow the fate of a cytotrophoblast differentiation antigen during the whole process of embryogenesis and the growth of the placental tissue.

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