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Dying and regeneration of human tumor cells after heterotransplantation to athymic mice

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Summary. The histologic phenomena occurring immediately after heterotransplantation of two human colon adenocarcinomas to athymic mice have been studied. The tumors differed with respect to velocity of growth and passage age. Three phases were discernible in both cases. (1) During the first phase, most inoculated tumor cells died. **(2)** The second phase was characterized by removal of the necrotic tumor cells by immigrated inflammatory cells and by penetration of the connective tissue of host animals from peripheral into central areas of the implants. The first mitoses occurred within tumor cells in close proximity to these connective tissue septa. **(3)** During the third phase, signs of regeneration and proliferation of tumor cells resulted in the macroscopic enlargement of xenografts. Only in this phase, the typical histologic characteristics of the tumors were formed.

These observations point to the host connective tissue invading into implants to be of great importance for the stimulation of tumor cell proliferation and, therefore, for the growth of xenografts. Thus, successful heterotransplantation is obviously based on mutual events between the transplanted tumor cells and host connective tissue.

Key words: Human colon adenocarcinomas - Heterotransplantation - Athymic mice - Histologic phenomena

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Introduction

During recent years, athymic nude mice have gained great significance for experimental oncology (Bastert et al., 1981). Because of the congenital absence of immunocompetent T-lymphocytes, they are generally unable to reject xenografts. In consequence, human tumors are often transplantable to nude mice in serial passages (Povlsen and Rygaard, 1971). Human tumors, which proliferate in nude mice, are comparable to metastases growing outside of human patients. They are easily accessible to numerous investigations which are not feasible in men. In this field, above all in pharmacologic studies within the scope of preclinical screening of newlydeveloped cytostatics against human tumors (Goldin and Wolpert-Defilippes, 1979; Osieka, 1984; Kleine, 1984; Kopf-Maier et al., 1985) though also in cytokinetic and morphologic studies, observing the cytologic effects of antiproliferative agents in human tumors (Azar et al., 1982; Kyriazis et al., 1983), new perspectives for experimental cancer research have been opened.

Whereas studies with pharmacologic purposes have been increasingly performed during recent years, scarce account has been taken of those phenomena occurring in human tumors heterotransplanted to nude mice without additional treatment. In the present study, the histologic alterations occurring after heterotransplantation have been studied in two human adenocarcinomas of the colon sigmoideum, differing from one another by velocity of growth and passage age.

Materials and methods

Animals

Male athyrnic mice (NMRI, nu/nu) were obtained from Gl. Bomholtgård Ltd, Ry, Denmark, and kept in isolators. The cages, bedding, food and water were autoclaved

before being placed in contact with nude mice. At the time of tumor transplantation, the animals were 8-10 weeks old and weighed about 18-20 g.

Tumors

Two human colon adenocarcinomas, designated CA1 and CA2, were used for the present investigations (Figs. 1,11). They differed from one another with respect to passage age and velocity of growth. Whereas CA1 was already in the 10th passage and grew rapidly (mean volume doubling time, 3.7 d), CA2 represented a rather slowly proliferating tumor (doubling time, 13.9 d) in the 2nd pasage. Both tumors derived from the colon sigmoideum, were characterized by a moderate degree of differentiation in the tissue of origin and did not show changes either of differentiation or of typical histologic features during passaging on the nude mice. CA2 was characterized by the presence of numerous well-developed adenoid structures, the lumina of which were mostly filled with plenty of necrotic cells and cellular debris (Fig. 11).

For heterotransplantation, the tumors were removed from donor animals when they had a size of about 1-2 cm^3. They were dissected and minced mechanically, mixed with two-fold volumes of minimum essential medium, which had been supplemented by fetal calf serum, streptomycin and penicillin, and pressed several times through injection needles with a diameter of 0.9 mm. Volumes of 0.3 ml of this tumor cell brei were then inoculated subcutaneously into the right flank of experimental animals. A group of 26 animals received tumor CA1, another 24 animals CA2. In the case of the rapidly growing CA1, the xenografts of every 2 animals were removed on days 1, 2, ..., 10, 12, 16, or 20 after transplantation and in the case of the slowly proliferating CA2, on days 1, 2, 4, 6, 8, 12, 16, 20, 24, 32, 40, or 48, respectively.

Preparation for light microscopy

After removal, all xenografts were cut in two parts and immediately immersed into fixative solutions. One half of the bisected tumors were fixed for 3 days in Bouin's solution (aqueous solution of picric acid, formaldheyde and glacial acetic acid), dehydrated and embedded in paraplast. For histologic examination, 2 to 4 µm thick sections were cut, mounted and stained with hematoxylin and eosin or with azocarmine and aniline blue (azan). The other group of tumor halves were fixed for 1 day in a solution containing 3% glutaraldehyde and 3% paraformaldehyde in cacodylate buffer (pH 7.2), dehydrated and embedded in Epon. Then, 1 µm thick sections were cut, mounted and stained with toluidine blue.

Results

Histologically, the tumor brei, which was injected, mainly consisted of single tumor cells (Figs. 2, 12). Clumps of cohering cells were seen only occasionally. In the case of CA1, only a few cells exhibited clear signs of cellular degeneration, some of the tumor cells even being found in a stage of mitosis at the time of inoculation (Fig. 2). Undulatory bundles of collagenous fibres were scattered sporadically between the tumor cells. Regarding the tumor brei of CA2, a greater number of necrotic cells occurred (Fig. 12), probably deriving from dead cells which were found peeled off into the adenoid lumina of the original tumors and of the xenograft of CA2 in the lst passage (Fig. 11).

Colon adenocarcinoma CA1

Whereas inoculated tumor cells were still mitotically active, the features of cellular dying predominated 1 day after transplantation of CA1. At the site of tumor inoculation, large agglomerations of tumor cells were found in all stages of cellular necrosis (Figs. 3,4). Only some tumor cells were obviously alive these being located in peripheral areas of the implant in close proximity to the subcutaneous connective tissue of the host animal (Figs. 3,4). Morphologically, they appeared rather inconspicuous, though they did not exhibit signs of proliferating activity. Swarming out of blood vessels in host subcutaneous tissue, crowds of inflammatory cells, especially leukocytes, penetrated into marginal zones of the xenografts (Fig. 3).

One day later, the implants mainly consisted of dying and dead cells and of numerous leukocytes and macrophages, which were about to phagocytose the necrotic tumor cells. At the periphery, some intact tumor cells were still present (Fig. 5). Additionally, few fibroblasts appeared as single cells in the most peripheral regions (Fig. 5) and obviously invaded into central areas of the xenografts.

On days 3 and 4 after transplantation, the fibroblasts were arranged in small chains, being embedded in fibrous, collagenous material (Fig. 6). These cords of connective tissue radiated from peripheral to central areas, sometimes contained capillaries and were surrounded by one or two layers of large, clear tumor cells. The latter showed marked nuclear polymorphism, often included intracytoplasmic lipid droplets, bud did not exhibit signs of ongoing necrosis (Fig. 6). The first mitoses always occurred within tumor cells immediately adjacent to the connective tissue cords (Fig. 8). Far from the immigrated stripes of connective tissue, especially in central areas of the implants, necrotic cells, amorphous cellular debris and inflammatory cells were still the most outstanding findings on days 3 and 4. Numerous macrophages were present, many of which contained large cytoplasmic inclusion bodies.

Another 2 days later, i.e. 5 and 6 days after transplantation, the tumors showed evidence of macroscopic growth. In all regions of the xenografts, there was a predominance of tumor cells containing only a few intracytoplasmic lipid droplets and showing no further signs of cellular degeneration (Fig. 7). The whole tumors were encircled by complete, thick capsules of connective tissue, from which septula penetrated into the interior of the tumors. The septula conducted numerous little blood vessels, especially capillaries (Figs. 7,8), and consisted of fibroblasts and plenty of collagenous fibres. The tumor cells in close proximity to connective tissue septa were often

Fig. 1. Human colon adenocarcinoma CA1 at the end of 9th passage on nude mice. Large tumor cell clusters, encircled by septa of connective tissue. Some adenoid structures (→). Azan, x 100

Fig. 2. Tumor brei of CA1 ready for inoculation. Some mitotic figures (→). Toluidine blue, x 960

Fig. 3. and 4. Heterotransplanted CA1, one day after inoculation. Large agglomerations of necrotic tumor cells, a few surviving tumor cells near host connective tissue (→), numerous inflammatory cells invading the xenograft (⇨). Azan, x 240, x 600

Fig. 5. CA1, 2 d after inoculation. Marginal zone of xenograft. Solitary intact tumor cells among necrotic cells. Some fibroblasts occurring as single cells (→). Toluidine blue, x 600

Fig. 6. CA1, 3 d after inoculation. Small cords of connective tissue invading the xenograft. Rather intact tumor cells immediatly adjacent to connective tissue. Only single tumor cell necroses. Toluidine blue, x 600

Fig. 7. CA1, 5 d after inoculation. Tumor cell cluster consisting of intact tumor cells and containing several mitotic figures. Around the tumor cell cluster, strands of connective tissue conducting some blood vessels. Azan, x 300

Fig. 8. CA1, 5 d after inoculation. Branching cord of connective tissue containing numerous capillaries and surrounded by regularly-arranged tumor cells. Several mitotic figures(➡)in close proximity to connective tissue. Toluidine blue, x 300

Fig. 9. CA1, 6 d after inoculation. Tumor cell cluster containing some mitotic figures (➡) and an adenoid structure in process of formation (⇨). Azan, x 600

Fig. 10. CA1, 8 d after inoculation. Adenoid structure encircled by a thin layer of connective tissue. Cellular debris in the lumen, luminally oriented brush border on the surface of tumor cells. Azan, x 600

Fig. 11. Human colon adenocarcinoma CA2 at the end of 1st passage on nude mice. A great number of necrotic cells within the adenoid lumina. Azan, x 100

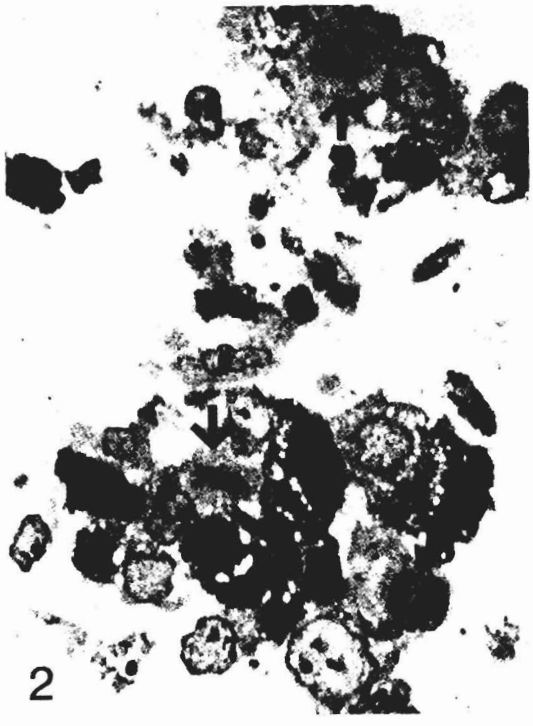
Fig. 12. Tumor brei of CA2. Numerous necrotic cells among morphologically intact tumor cells. Toluidine blue, x 960

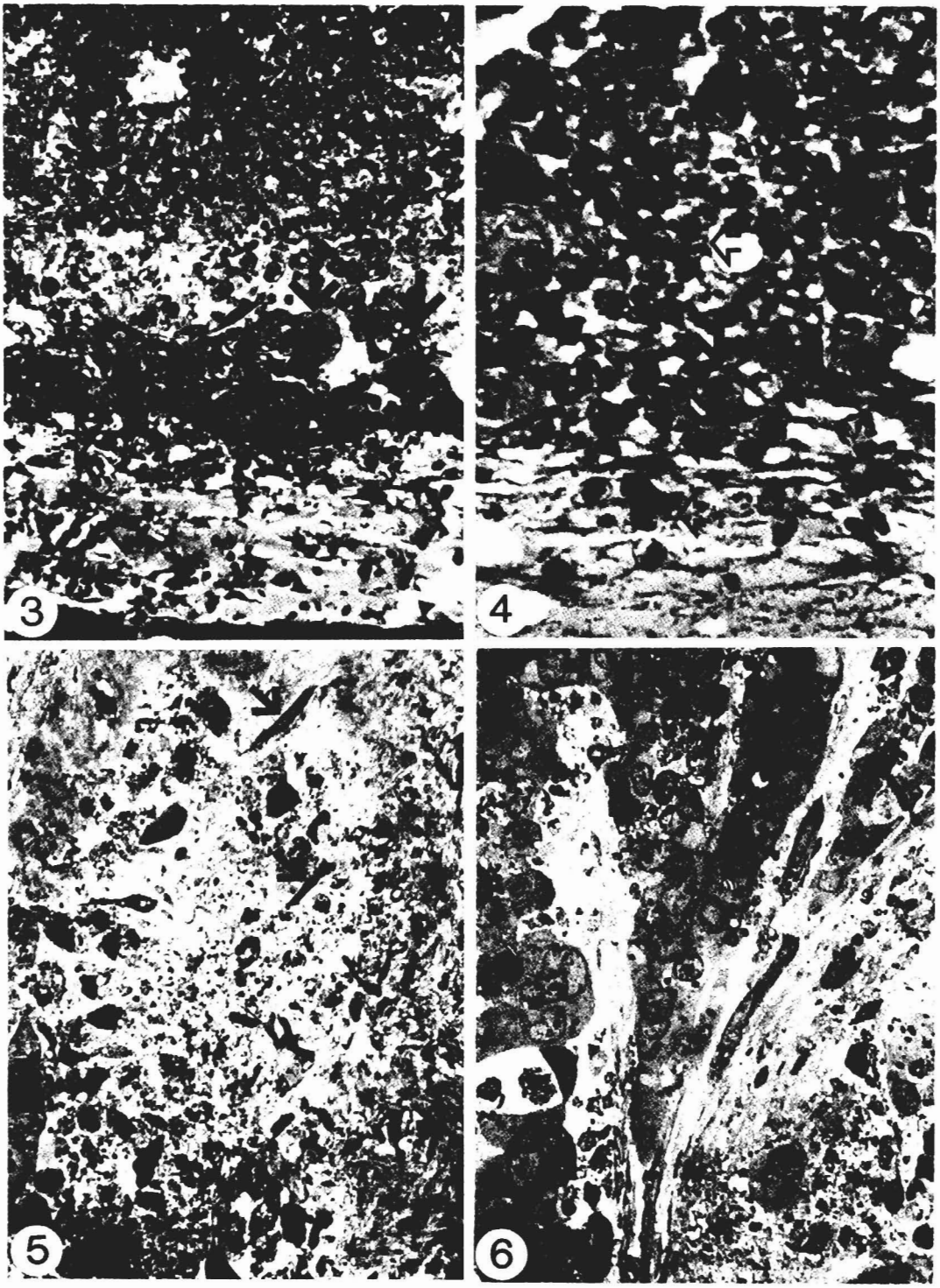
Fig. 13. CA2, 2d after inoculation. Accumulations of necrotic cells (N) invaded by crowds of inflammatory cells. A group of fairly intact tumor cells near mouse connective tissue. (➡). HE, x 190

Fig. 14. CA2, 6 d after inoculation. A wedge-shaped cord of connective tissue invading an accumulation of necrotic tumor cells (N). Small clusters of fairly intact tumor cells in close proximity to connective tissue. HE, x 240

Fig. 15. CA2, 8 d after inoculation. Some clusters of morphologically intact tumor cells embedded in mouse connective tissue. Mitotic figure (➡). HE, x 190

Fig. 16. CA2, 20 d after inoculation. Large cluster of tumor cells, encircled by an envelope of connective tissue and containing several adenoid structures. Mitotic figures(➡). Azan, x 150

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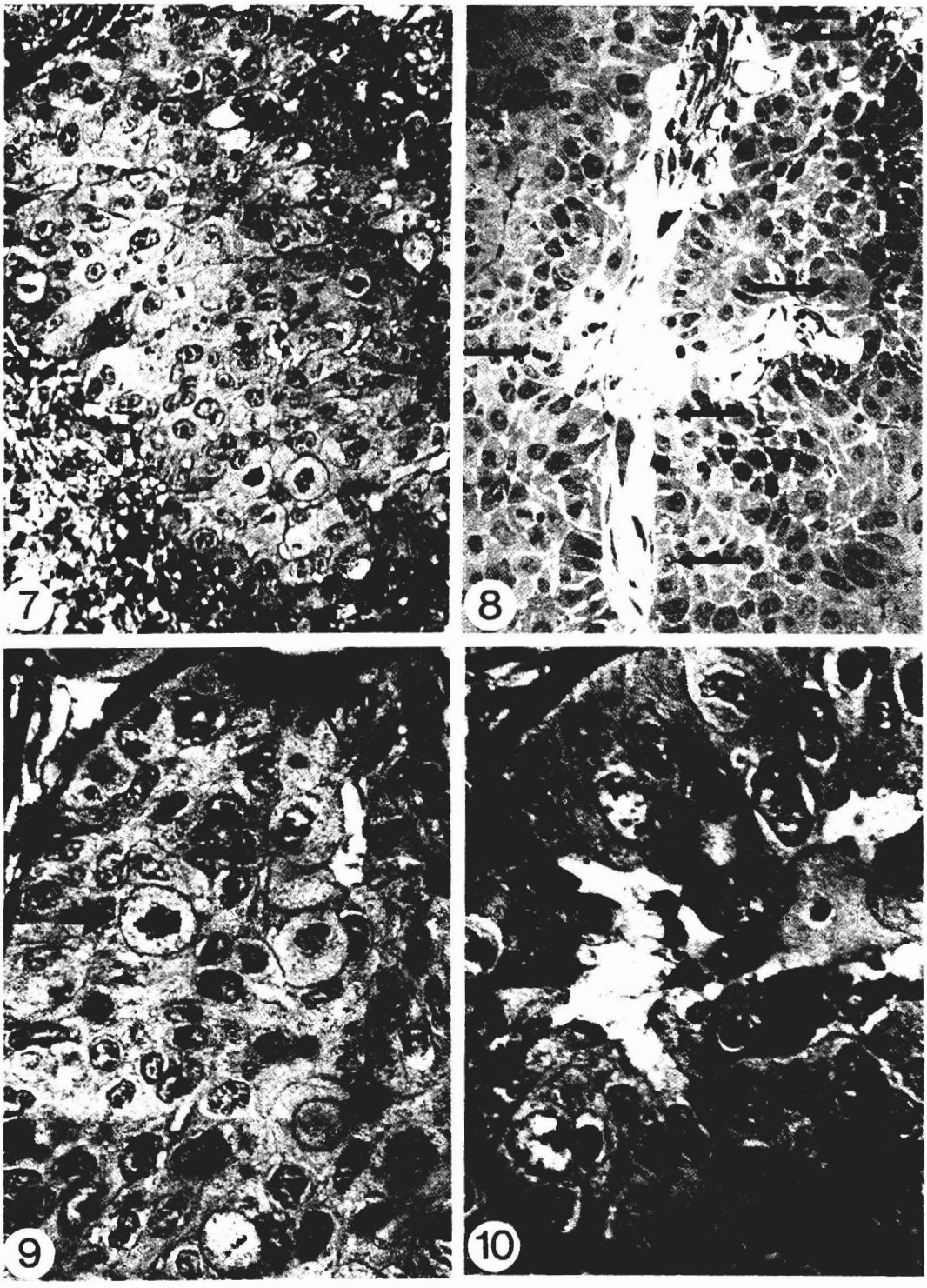
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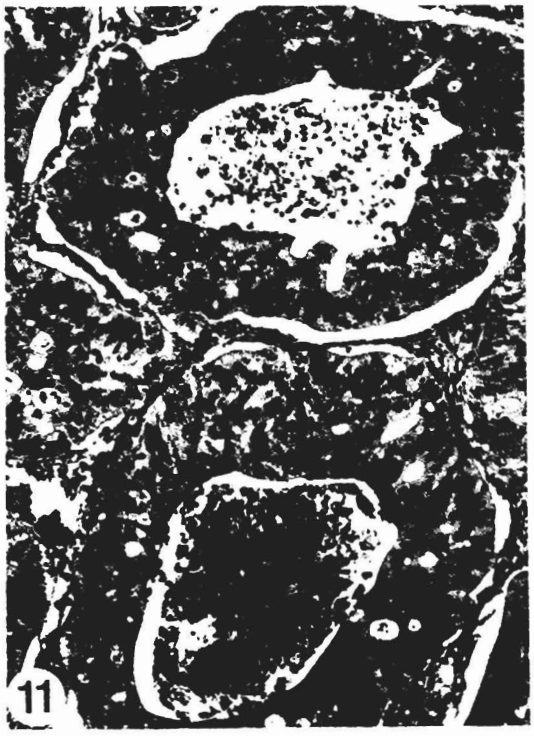

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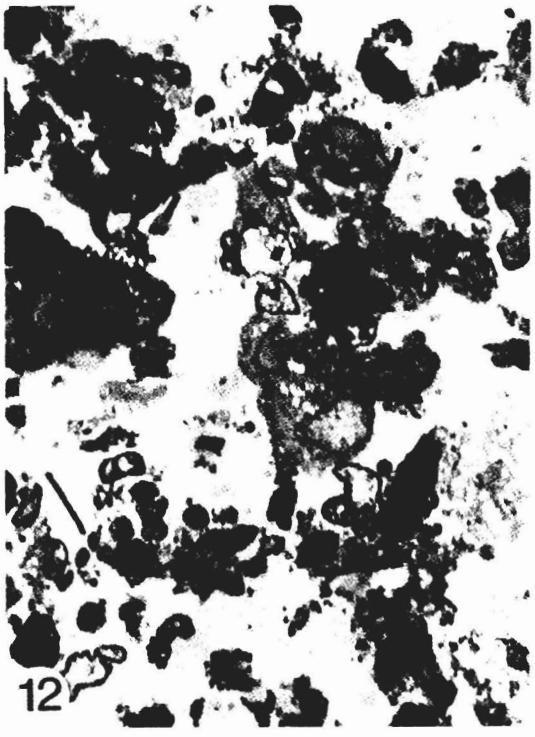

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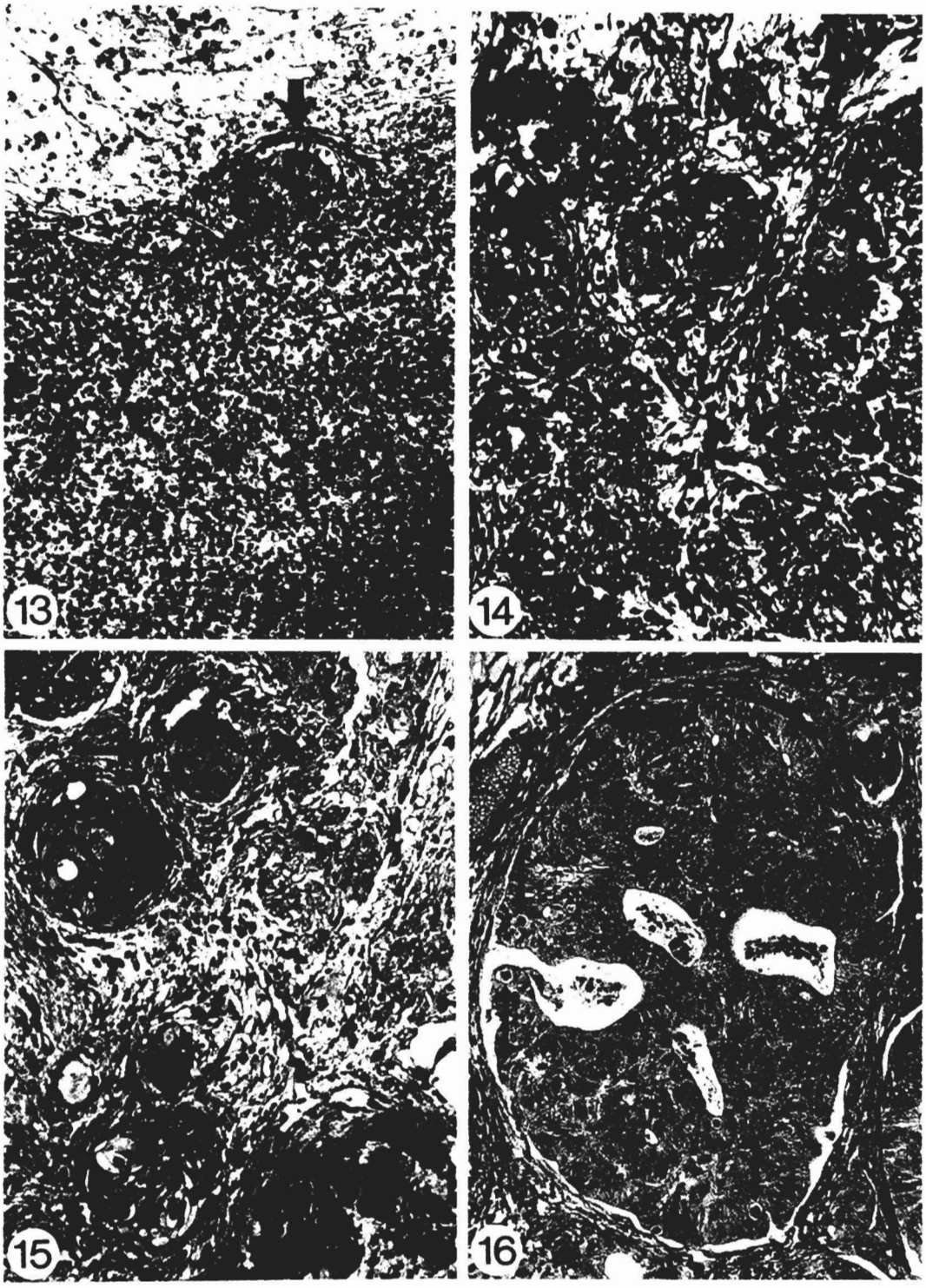
regularly lined up side-by-side and contained numerous mitotic figures (Figs. 8, 9). On day 6, the first adenoid structures appeared (Fig. 9), surrounded by a mostly incomplete and thin connective tissue envelope and consisting of two or three layers of tumor cells. These were arranged circularly around a central lumen and often bore a luminally oriented brush border. The lumen itself was either empty or contained mucous material and cellular debris (Fig. 10).

These morphologic developments intensified during the following 2 days. Thus, on days 7 and 8, only solitary tumor cell necroses and only a few inflammatory cells occurred. Large and clear tumor cells without degenerative signs predominated. They were assembled in bulky balls encircled by big septa of connective tissue. The latter contained numerous and voluminous blood vessels. Many tumor cells were about to divide and numerous adenoid structures were present. These results confirm that, on days 7 and 8, the histologic appearance of the heterotransplanted tumor CA1 was identical to that at the end of the preceding passage. It remained unaltered until the end of the experimental period, i.e. until day 20.

Colon adenocarcinoma CA2

In the case of the more slowly-growing tumor CA2, the same morphologic events were, in principle, observable; the time parameters, however, differed from those of CA1. Most of the CA2 tumor cells, which were inoculated, also died within 1-2 days after transplantation (Fig. 13). Beginning on day 1, inflammatory cells invaded the xenografts (Fig. 13). Fibroblasts appeared in marginal zones of the implants of day 4; cords of connective tissue, radiating from the periphery into the interior of the implants, were formed between days 4 and 8 (Fig. 14) and the tumors were entirely encircled by collagenous envelopes on day 12 after transplantation. On day 6, the first mitoses of the tumor cells appeared and, on day 8, small spherical tumor cell clusters were found in contact with the cords of connective tissue within the xenografts (Fig. 15). In most cases, these tumor cell clusters did not contain a central lumen and consisted of morphologically fairly intact tumor cells. On day 16, the removal of dead tumor cells and cellular debris by inflammatory cells had apparently finished (Fig. 16); macroscopic growth of the tumors became evident from day 12. Numerous adenoid structures appeared on day 12; their lumina were filled up with large amounts of dead cells and cellular debris between days 16 and 24 (Fig. 16). On day 24, the morphologic characteristics of CA2 in the 2nd passage on nude mice were identical to those at the end of the lst passage and remained unaltered until the last day of investigation.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Discussion

Three successive phases, flowing into each other, are discernible after heterotransplantation of human colon carcinomas into athymic, congenitally immunodeficient mice.

(1) First of all, the results of the present study underline that proliferation of xenografted tumor cells does not begin immediately after transplantation into nude mice. In contrast, most of the inoculated cells die during the first days after transplantation and only a few tumor cells survive. This result is unexpected with respect to the apparent morphologic integrity and the obviously retained proliferation activity of inoculated human tumor cells. On the other hand, the phenomenon of cellular dying corresponds to the findings observed, e.g., after primary in-vitro-cultivation of mammalian cells (Mauersberger, 1971; Salmon, 1980). Environmental changes and, perhaps, insufficient nutritional conditions may be responsible for cell death in the case of heterotransplantation of human tumor cells to nude mice.

(2) During a second phase, processes of stabilization predominate. The necrotic tumor cells are removed by inflammatory cells belonging to the host defensive system. Simultaneously, fibroblasts penetrate from the subcutaneous connective tissue of host animals and form cords of connective tissue within xenografts. They contain numerous blood vessels and are soon surrounded by fairly intact tumor cells showing vivacious proliferative activity. Investigations by other authors using methods of immunofluorescence (Warenius, 1980) and histochemistry (Fiebig and Löhr, 1984), have clearly confirmed that the cords of connective tissue, blood vessels and blood cells are not of human origin in heterotransplanted tumors, but derive from host animals. These elements are certainly important for the nutrition of heterotransplanted tumors. Moreover, the results of the present study suggest that the invading host connective tissue may also play a key role in the induction of tumor cell proliferation and, thus, in tumor survival and development.

(3) During a third phase, tumor cell regeneration and proliferation result in macroscopic and continuous enlargement of heterotransplanted tumors. It is worth mentioning that it is only in this phase that the typical histologic features of the tumors are formed. In consequence, it is ingenious to perform morphologic, cytokinetic or pharmacologic studies with heterotransplanted tumors only at this point.

These three phases are apparently a constant pattern after xenografting human tumors, at least human colon carcinomas, to athymic mice and occur independently of growth velocity and passage age of the xenografts. The time parameters, however, are different and depend on individual conditions, especially on the individual growth velocity of tumors in nude mice.

The results of the present study underline that the success of heterotransplantation is not only influenced by the proliferation activity of inoculated tumor cells, but also by a timely penetration of the host connective tissue into xenografts and, probably, by an interaction between elements of host connective tissue and tumor cells. Ultrastructural studies are necessary to gain further insights into these interaction processes, being indispensable to resolve whether heterogenous types of cells are differently involved in the processes of cell death and cellular regeneration.

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