HETEROLOGOUS TRANSPLANTATION OF A TISSUE CULTURE LINE OF A HUMAN MELANOMA (TC491)**

It is both difficult and often impractical to obtain viable human tumors at the convenience of the investigator. For a variety of biochemical, biological, and pharmacological studies, significant quantities of neoplastic tissues are required. Primary animal tumors are used preferentially by investigators. The techniques of heterotransplantation of human neoplasms, so successfully used by the late Harry S. N. Greene, offer at least as many advantages as experimentation with primary animal tumors. Furthermore, objections expressed regarding the extrapolation of results from experimental animal melanomas to the human situation may be overcome to a large extent by using human heterotransplants.

This article reports a study of a human melanoma, maintained as a long-term tissue culture line and subsequently transferred to the cerebrum of healthy unconditioned guinea pigs. The fate of the neoplastic cells was followed up from passage one to higher passages. The dynamic properties of these neoplastic cells were observed as they adapted to the environment in vitro, and as they subsequently expressed themselves when transferred to the living host.

MATERIAL AND METHODS

The human tumor used for this study was obtained from the Department of Neurosurgery, Hartford Hospital, Hartford, Connecticut, and represented a metastasis of a melanotic melanoma to the cerebellum. The tumor was transported to New Haven under sterile conditions in a Petri dish containing gauze moistened with saline. Immediately after receiving the specimen, parts of the tumor were used for tissue culture purposes and other portions were transplanted into the eyes of healthy unconditioned guinea pigs; the result of the latter experiment will be reported elsewhere.

Preparation of tissue cultures: A technique of initiation and propagation of long-term tissue culture developed in this laboratory and reported in detail elsewhere was utilized. Solid portions of melanotic tumors were minced with small scissors in a Petri dish. Small quantities of the fragments were distributed to three 3-oz. Brockway bottles. Approximately 10 cc. of nutrient fluid containing Eagles medium, 10% noninactivated serum, 1% glutamine and penicillin (100 units/ml.) and streptomycin (100 mg/ml.) were added to each bottle. The bottles were closed with rubber stoppers and placed in a walk-in incubator maintained at 37°C. The tissue cultures were read every 24 hours. When growth on the glass surface became luxurious, covering 100% of the surface, the bottles were trypsinized and subdivided.

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The generation time of the tissue cultures was determined by nuclei count, according to Rappaport's modification of the method of Parker, et al. From healthy cultures in the log phase, 36 3-oz. Brockway bottles were prepared, each containing approximately 300,000 cells suspended in 10 cc. of medium with 10% horse serum. Beginning at 24 hours and every 12 hours thereafter, four bottles were taken for nuclei count.

Heterologous transplantation of tissue culture lines: A method of heterologous transplantation of long-term tissue culture lines developed by us and reported in detail elsewhere was utilized. Two hamsters received total x-irradiation with 250 r. They were then injected with 4.5 mg. of cortisone acetate on days 0, 2, and 3. Each hamster received subcutaneously 0.5 cc. of melanoma tissue culture cells in medium. The solution was prepared by adding 0.25 cc. of closely packed tissue culture cells to 0.75 cc. tissue culture medium. When subcutaneous tumors developed, the animals were sacrificed. The subcutaneous tumor was removed steriley and transferred heterologously into the brains of healthy, unconditioned guinea pigs.

RESULTS

Tissue cultures: The culturing of the melanotic melanoma (TC491) was initiated on April 26, 1963. Generally, the morphologic appearance of the tissue culture cells, the presence or absence of melanin pigment, and the rate of growth of cells growing in monolayer fashion, were assessed. These characteristics varied with 1) the time at which the cells were microscopically examined after initiation of line TC491 and, 2) the time interval at which each subculture was microscopically evaluated, e.g., 24 hours, 48 hours, etc.

The tissue culture cells were markedly melanotic. The medium and supernatant fluid were brown-black due to the presence of melanin pigment and melanin was present both in tumor as well as phagocytizing cells. The initial cultures were observed only in the living state and were not fixed so that they all could be subcultured. When cells covered 100% of the surface of the bottle, subcultures were prepared. In cultures during the periods of initiation of the line round, melanin-bearing macrophages were seen occasionally. The number of fibroblasts and macrophages was small early in the initiation of line TC491 and these cells became fewer in subsequent subcultures. At times it was difficult, if not impossible, to discriminate between bipolar melanocytes and fibroblasts.

Permanent histological preparations from later subcultures of the line were made from small coverslips put into Leighton tubes and these were prepared and examined at varying intervals after initiation of each subculture. Coverslips examined 24 hours after subculturing revealed a variety of pleomorphic cells with no melanin pigment (Fig. 1). Most of the cells grew in an isolated fashion. Some cells were round or ovoid and others had stellate, bipolar or triangular dendritic forms. Some cells had bulbous tips, others long variegated processes. Mitotic figures, giant nuclei and giant cells
were seen occasionally. The dendritic forms resembled glial or neuronal
cells in culture.

At later intervals after the initiation of the subculture (2 days or more)
cells, mostly round to triangular, were seen growing in small accumulations
of clusters. A definite increase of polydendritic forms was seen along with
an increasing number of pigmented cells. Some round forms resembling
lymphocytes remained, which were difficult to discriminate from the pig-
mented cells. In older subcultures (4 days or more) many cells had melanin
pigment. Generally speaking, the older the subculture the more pigment was
seen, and the size of the individual cells was larger.

The morphologic appearance of tissue culture cells was also dependent
upon their rate of growth. In fast growing cultures the cells appeared to
grow in more than one layer. When the tissue culture cells became crowded,
their cytoplasmic processes became less visible (Fig. 2). Such cultures had
a more epithelial-like appearance, and only at the periphery of a heavy and
luxuriant growth could one still recognize the morphological variations of
the melanoma cells.

One cell type of line TC491 was characterized by unusual morphological
features including its monstrous size of 100 μ or more. Some of these cells
contained in their cytoplasm one or more melanocytes. The latter often
seemed intact with well preserved cell membranes and without any regres-
sive changes. The incorporated cells were separated from the cytoplasm of
these monstrous cells by a clear halo (Fig. 3). Some of these unusual cells
were bi- or multinucleated.

The rate of growth of TC491 was determined on September 16, 1963,
five months after its initiation. As one can see in Figure 4, the generation
time of this culture is approximately 36 hours.

In early 1964, the culture became almost amelanotic; with this event a
decrease of the generation time was detected. The cells grew faster, yet
their morphologic appearance was similar to that of the melanin-bearing
cells except for the absence of the pigment. Thus, bipolar, triangular, stel-
late, round, ovoid and/or epithelial-like cells were seen in the amelanotic
cultures identical in appearance to those described in the melanotic variety.
When the subcultures of this long-term amelanotic line TC491 were
crowded, however, the cells were predominantly epithelial-like, and only
when the cells grew in isolated fashion were pleomorphic forms seen.

Line TC491 died on November 26, 1969 due to an increase of the tem-
perature in the walk-in incubator as a result of failure of the thermostat. It
had been growing for 343 subcultures.

Heterologous transplantation: The subcutaneous transplantation of line
TC491 occurred on August 19, 1963, four months after initiation of this
### Table 1. Heterologous Transplantation of N 491

| Passage no. | +/- takes (+/total animals) | Percent positive | Interval between transplantation and neurological signs (Days) |
|-------------|-----------------------------|------------------|---------------------------------------------------------------|
| 1           | 2/5 (2/5)                   | 40               | 70,70                                                         |
| 2           | 5/20 (5/24)                 | 25               | 44,43,55,63,75                                               |
| 3           | 3/12 (3/12)                 | 25               | 45,67,72                                                     |
| 4           | 8/14 (8/15)                 | 57.1             | 26,31,35,39,47,47,47,77                                       |
| 5           | 4/10 (4/12)                 | 40               | 39,46,49,48                                                 |
| 6           | 8/10 (8/12)                 | 80               | 22,25,27,33,34,34,34,48                                      |
| 7           | 5/10 (5/10)                 | 50               | 34,44,49,58,58                                              |
| 8           | 5/8 (5/9)                   | 62.5             | 49,52,52,55,63                                              |
| 9           | 3/5 (3/9)                   | 60               | 46,53,56                                                    |
| 10          | 3/6 (3/8)                   | 50               | 55,55,58                                                    |
| 11          | 5/8 (5/9)                   | 62.5             | 37,44,50,56,74                                              |
| 12          | 3/10 (3/10)                 | 30               | 40,49,61                                                    |
| 13          | 7/8 (7/10)                  | 87.5             | 35,49,54,55,55,62                                           |
| 14          | 9/9 (9/10)                  | 100              | 40,41,41,48,50,54,63,72,79                                  |
| 15          | 2/10 (2/10)                 | 20               | 52,59                                                       |
| 16          | 4/7 (4/9)                   | 57.1             | 28,38,40,80                                                 |
| 17          | 6/8 (6/10)                  | 75               | 25,43,60,60,60,60,60                                         |
| 18          | 6/9 (6/10)                  | 66.6             | 21,45,45,59,59,59                                          |
| 19          | 3/8 (7/11)                  | 37.5             | 42,49,49,63,66,80,80                                         |
| 20          | 3/10 (3/10)                 | 30               | 54,55,81                                                    |
| 21          | 7/10 (7/10)                 | 70               | 32,32,32,40,41,42                                           |
| 22          | 6/10 (6/10)                 | 60               | 19,21,29,35,36,36                                          |
| 23          | 4/9 (4/10)                  | 44.4             | 18,25,25,25                                                |
| 24          | 3/7 (3/10)                  | 42.8             | 24,24,24                                                   |
| 25          | 4/7 (4/9)                   | 57.1             | 27,27,29,29                                               |
| 26          | 2/2 (2/10)                  | 100              | 32,38                                                      |
| 27          | 5/9 (5/10)                  | 55.5             | 25,26,26,26,26                                             |
| 28          | 6/8 (6/10)                  | 75               | 22,28,34,34,34,34,37                                        |
| 29          | 3/8 (3/10)                  | 37.5             | 38,52,52                                                   |
| 30          | 4/10 (4/10)                 | 40               | 18,21,35,56                                                |
| 31          | 7/8 (7/10)                  | 87.5             | 17,24,24,26,27,28,31                                       |
| 32          | 6/9 (6/10)                  | 66.6             | 31,31,31,32,35,48                                         |
| 33          | 8/10 (8/10)                 | 80               | 25,26,26,30,32,35,36,37                                    |
| 34          | 6/10 (6/10)                 | 60               | 22,22,25,27,33,35                                         |
| 35          | 9/10 (9/10)                 | 90               | 24,29,30,31,31,31,31,32,36                                 |
| 36          | 8/9 (8/10)                  | 88.8             | 18,20,22,28,34,34,35,42                                     |
| 37          | 8/10 (8/10)                 | 80               | 23,25,27,28,28,28,33,35                                    |
| 38          | 5/9 (5/10)                  | 55.5             | 21,29,36,39,40                                             |
| 39          | 5/9 (5/10)                  | 55.5             | 24,24,26,28,40                                             |
| 40          | 5/8 (5/9)                   | 62.5             | 22,22,22,27,28                                           |
| 41          | 6/9 (6/10)                  | 66.6             | 22,23,23,23,23,26                                         |
| 42          | 6/8 (6/9)                   | 75               | 18,20,24,24,24,24,24                                       |
| 43          | 5/9 (5/9)                   | 55.5             | 22,22,25,25,26,32                                         |
line. Several tissue culture cells were still melanotic. Subcutaneous tumors developed eleven days later in the conditioned hamsters and these neoplasms were in turn transferred to the brain of healthy unconditioned guinea pigs. Two out of five animals showed large intracerebral melanotic tumors (N491) 70 days later. Part of the neoplasm was fixed for histological examination and other parts were transplanted to the brains of healthy guinea pigs. Neoplasm N491 has been transferred from healthy host to healthy host up to the present time, that is, for the past seven and one-half years. Since the heterologous tumors grew equally well in both sexes, males and females were kept in the same cages. Guinea pigs love sex too.

Table 1 shows the transplantation behavior of this tumor. The number and percentage of positive takes are indicated for each passage. The numbers in parentheses indicate the total number of animals used for each passage. Since, however, some animals died very early after the transfer of the tumor, due to trauma, infection, or unknown reasons, all those that died within 10 days of the transplantation of the tumor were eliminated from our calculations. In the last column, the time in days between transplantation and the
development of neurological signs for each positive take in each passage is given. The number in italics designates the animal whose tumor was transferred in the subsequent passage. Without using any elaborate statistical analysis it is evident that after approximately the 21st passage, the hetero-transplants grew faster. Although 65 passages were evaluated in the present study, the tumor is in its 80th passage at present time. It is a very fast growing tumor and most of the animals developed neurological signs approximately 22-25 days after inoculation. Twenty-four hours prior to death the positive animals showed motor weakness, paralysis, tremors, and occasionally convulsions and opisthotonus. Some guinea pigs appeared agitated and moved in circles. Shortly before death, positive animals lay on their sides with generalized tremors.

During the 7th passage of the tumor, one positive animal developed a neoplasm that was grossly amelanotic with only a few dark regions in the center of the large tumor. From the light parts of the growth a subline was initiated, indicated as N491 Light (see Table 2). The tumor N491 Light grew somewhat faster than N491. Grossly, this subline was amelanotic. Histologically, in early transfers a limited number of melanotic cells was present. After a few heterologous passages N491 Light became totally amelanotic. The histology of this subline was very similar to that of N491 except for the absence of melanin pigment and the accelerated rate of growth. After this line was observed for 33 passages, it was arbitrarily terminated.

In other animals from passage 7 of line N491 the gross appearance of the tumor was black. In subsequent passage of these, however, the gross appearance of the tumor varied from pitch black to red. It should be emphasized that the number of melanotic cells observed microscopically did not always coincide with the gross appearance of the tumor, e.g., grossly red-gray appearing tumors revealed, microscopically, several melanotic cells. At the present time the gross appearance of the tumors varies between gray-red and white.

The size of the intracerebral tumors varied, although, as a rule, the tumors were very large; they occupied one hemisphere and often grew contralaterally to involve the other hemisphere, as is seen in a photomicrograph from passage 9 (Fig. 5). Microscopically this tumor had a carcinomatous appearance with marked cellular pleomorphism. No particular cytoarchitectural pattern was seen in most of the passages (Fig. 6). The size of the individual cells varied considerably and many giant nuclei and giant cells were seen in some parts of a given tumor and in some passages in particular (Fig. 7). As a rule, most of the nuclei had a distinct nucleolus. The amount of cytoplasm varied considerably. Also, the amount of melanin varied from
cell to cell and from region to region in a given tumor. Huge giant cells, some of which clearly contained melanin pigment, also were seen (Fig. 8). On the other hand sometimes small, hyperchromatic, markedly anaplastic and undifferentiated tumor cells could be seen nearby. In addition, in some tumors and in some regions of a given tumor, numerous monster giant cells that appeared sometimes to “phagocytize” or to incorporate smaller melanoma cells (Figs. 9 and 10) were seen. Rarely, some elongated forms of

### Table 2. Heterologous Transplantation of N 491 Light

| Passage no. | +/−takes (+/total animals) | Percent positive | Interval between transplantation and neurological signs (Days) |
|-------------|---------------------------|-----------------|-------------------------------------------------------------|
| 1           | 7/10 (7/10)               | 70              | 13,21,24,27,38,38,42                                        |
| 2           | 9/12 (9/12)               | 75              | 26,27,29,29,29,36,36,43,56                                   |
| 3           | 5/8 (5/10)                | 62.5            | 20,25,25,25,30                                               |
| 4           | 8/8 (8/10)                | 100             | 20,20,21,21,21,21,21                                         |
| 5           | 5/8 (5/10)                | 62.5            | 22,25,35,35,35,42                                            |
| 6           | 7/10 (7/10)               | 70              | 20,25,25,27,35,40,48                                         |
| 7           | 7/9 (7/10)                | 77.7            | 18,19,19,21,25,27,27                                         |
| 8           | 4/7 (4/10)                | 57.1            | 21,26,26,40                                                  |
| 9           | 2/3 (2/10)                | 66.6            | 20,20,30,30,30,35                                            |
| 10          | 6/8 (6/10)                | 75              | 18,25,30,30,30,49                                            |
| 11          | 4/9 (4/10)                | 44.4            | 20,20,21,21,21,21,21                                         |
| 12          | 5/9 (5/10)                | 55.5            | 22,25,28,29,29                                               |
| 13          | 4/5 (4/10)                | 80              | 20,20,22,29                                                  |
| 14          | 9/9 (9/10)                | 100             | 21,23,24,24,24,24,25,25,29,30                               |
| 15          | 4/9 (4/10)                | 44.4            | 18,22,32,32,50                                               |
| 16          | 5/8 (5/10)                | 62.5            | 20,23,28,30,50                                               |
| 17          | 5/10 (5/10)               | 50              | 15,18,18,21,21                                              |
| 18          | 4/5 (4/10)                | 80              | 22,32,33,39                                                 |
| 19          | 1/10 (1/10)               | 42              | 20,25,25,25,27,27,27,27                                      |
| 20          | 7/10 (7/10)               | 70              | 21,22,22,28,34,35,35                                         |
| 21          | 8/9 (8/10)                | 88.8            | 19,20,25,25,26,27,27,27,37                                  |
| 22          | 3/5 (3/10)                | 60              | 21,21,21                                                   |
| 23          | 1/2 (1/10)                | 50              | 27                                                          |
| 24          | 5/9 (5/9)                 | 55.5            | 26,29,29,29,34                                               |
| 25          | 2/8 (2/9)                 | 25              | 27,39                                                      |
| 26          | 6/8 (6/10)                | 75              | 20,25,29,32,34,34                                            |
| 27          | 5/10 (5/10)               | 50              | 26,26,27,42,46                                              |
| 28          | 7/8 (7/10)                | 87.5            | 19,20,21,22,28,34,39                                       |
| 29          | 7/10 (7/10)               | 70              | 18,21,23,24,25,30,33                                        |
| 30          | 6/10 (6/10)               | 60              | 16,19,19,23,23,23,27                                    |
| 31          | 1/7 (1/10)                | 14.2            | 29                                                          |
| 32          | 1/5 (1/10)                | 20              | 25                                                          |
| 33          | 1/10 (1/10)               | 10              | 37                                                          |
tumor cells appeared that did not show any particular arrangement in bundles (Fig. 11); such parts always were reticulum negative. Occasionally, in regions of necrosis, perivascular infiltrates, composed predominantly of lymphocytes, were seen. In several passages, but especially in fast growing transplants, necrotic foci were seen with marked activity of phagocytizing cells (Fig. 12). Melanin pigment was easily detectable and evident in some of these necrotizing lesions (Fig. 13). Occasionally, around such regions of necrosis, a pseudopalisading arrangement of preserved tumor cells around a necrotic focus was evident (Fig. 14). The tumors were well vascularized, and occasionally one could see mild endothelial proliferation. The Laidlaw connective tissue stain demonstrated only very fine stromal septa and no reticulum fibers in the solid parts of the tumor (Fig. 15) with the exception of regions of scarring. While the tumor in some parts was well demarcated from the surrounding normal brain tissue (Fig. 7), in other parts the tumor was seen infiltrating normal structures (Fig. 16). Around the tumor in the normal tissue one could detect mild proliferation of both microglia and astrocytes. Many mitotic figures were seen in some fields (Fig. 17). With the passing of time and from passage to passage, the general histological appearance of the tumor did not change (Fig. 18). The only major change was the decrease of melanin to negligible amount.

Up to the 21st passage N491 was moderately melanotic. Melanotic cells occurred in small circumscribed regions of well preserved parts of the tumor, but, also concentrations of pigmented cells—mostly macrophages—were seen in parts of the neoplasm showing regressive changes. From the 22nd passage on, the melanin content of the heterotransplant was detectable in very small amounts and in very few or isolated cells. In subsequent passages the melanin content of tumor cells was negligible and one had to search in many fields before discovering melanotic cells. After the 57th passage no melanin pigment was seen in N491.

**DISCUSSION**

In recent years several studies have been reported on the behavior of human and animal melanomas in vitro, with excellent reviews of the pertinent literature.\(^1\)\(^-\)\(^9\) Rosenberg, *et al.* stated that melanotic and amelanotic variants of hamster melanoma in vitro have several characteristics identical with those of human melanoma tumors.\(^1\)\(^4\) Comparison of live TC491 with above mentioned cultures and with tissue cultures of other animal melanomas, e.g., Cloudman melanoma, convince us that the cellular morphology is very similar in cultures arising from animal and human tumors. The majority of the reports deal with short term tissue cultures. In early subcultures before becoming a long-term line, the cellular morphology of the TC491 was similar
FIG. 1. TC491. Bipolar, triangular and polygonal tissue culture cells are seen, some with long slender processes. Hematoxylin eosin. ×500.

FIG. 2. TC491. Fast growing crowded tissue culture cells, most of which have an epithelial-like appearance. In the background long, slender processes are seen. Individual cells have a polydendritic appearance. Phase contrast. ×300.
Fig. 3. TC491. A large cell is seen incorporating a relatively well preserved melanocyte. A clear halo is present around the melanocyte. Hematoxylin eosin. X500.

Fig. 4. Generation time of TC491 as determined by nuclei counts at varying time intervals.
Fig. 5. N491. Passage 9. A large tumor is seen occupying one hemisphere and growing contralaterally. Hematoxylin eosin. ×7.5.

Fig. 6. N491. Passage 2. A markedly pleomorphic tumor is seen with variations in the size of the nuclei and cells. Most of the nuclei contain a nucleolus. Giant nuclei and multinucleated giant cells are present. No particular cytoarchitectural arrangement is seen. Hematoxylin eosin. ×250.
Fig. 7. N491. Passage 7. The periphery of the tumor is characterized by markedly pleomorphic cells. A large number of giant cells is present. The tumor is well demarcated from the surrounding brain tissue. Hematoxylin eosin. ×250.

Fig. 8. N491. Passage 7. Markedly anaplastic tumor is present. In the center a giant cell is seen with melanin pigment in the cytoplasm. The chromatin content of the nuclei varies. Hematoxylin eosin. ×550.
Fig. 9. N491. Passage 7. Several giant cells are seen. In the center of the figure one monstrous cell is seen incorporating in its cytoplasm three melanocytes. To the left, acidophilic inclusion-like structures are seen in the nuclei of a giant cell. Melanin pigment is seen freely in the tissue and in the cytoplasm of many giant cells. Hematoxylin eosin. X430.

Fig. 10. N491. Passage 7. The center is occupied by a monstrous, binucleated cell in the cytoplasm of which many neoplastic melanocytes are included. Some of the cells are separated from the cytoplasm by a clear halo. Hematoxylin eosin. X430.
Fig. 11. N491. Passage 20. Many elongated or ovoid cells are seen with hyperchromatic nuclei. No particular arrangement in bundles is seen. Hematoxylin eosin. ×320.

Fig. 12. N491. Passage 25. A region of necrosis is seen and at the periphery, many phagocytizing cells. In the well-preserved parts of the tumor two multinucleated giant cells are present. Hematoxylin eosin. ×500.
FIG. 13. N491. Passage 20. In a region of the tumor revealing regressive changes, masses of melanin are seen in macrophages or free in the tissue (lower half of figure). Hematoxylin eosin. X320.

FIG. 14. N491. Passage 25. Pseudopallisading of the preserved tumor cells is seen around a focus of necrosis. Vacuolization is present in the peripheral part of the tumor. Hematoxylin eosin. X250.
Fig. 15. Delicate reticulum septa are seen separating islands of tumor cells. Laidlaw connective tissue stain. $\times 600$.

Fig. 16. Passage 4. Infiltration of the normal brain tissue is seen by markedly anaplastic tumor cells. Hematoxylin eosin. $\times 250$. 
Fig. 17. N491. Passage 52. An anaplastic tumor with five mitotic figures is seen. Some of the tumor cells have distinct nucleoli. Hematoxylin eosin. ×500.

Fig. 18. N491. Passage 63. No change in the morphologic appearance in this late passage is seen in comparison with early passages (see Fig. 6, Passage 2). Giant nuclei and giant cells are seen among the pleomorphic tumor cells. Hematoxylin eosin. ×500.
to that reported by others. The dynamic properties of these cells growing in vitro were expressed by their morphological variations, the presence or absence of melanin, variations in their incidence of mitoses, and generally, changes in their rate of growth. At the initiation of TC491, in addition to the neoplastic cells, small numbers of fibroblasts and macrophages were detected; however, in our monolayer cultures they decreased in number during early subculturing. The melanoma cells revealed marked pleomorphism including bipolar, tripolar, round, ovoid, polydendritic and epithelial-like forms. Several cells showed variegated processes and still others had bulbous tips. As in the study by Cobb and Walker,7 detached tips of cytoplasm were observed in the vicinity of cells and these were probably a result of clasmatosis.8 Furthermore, the dendritic forms of melanoma cells resembled nervous tissue in culture, as other workers have stated.7 Indeed, it was striking to note the resemblance of the dendritic melanocytes to the stellate and dendritic cells of glioma cultures propagated in our laboratory. In a careful study, Hu reported variations in the appearance of melanocytes at different time intervals, e.g. 24 hours, 3-5 days etc.; melanin was absent in the first 48 hours and started appearing in isolated cells 4 days after initiation of the subculture.9 Similar observations were made with TC491 while the line was still melanotic. It seems that melanin formation in vitro depends upon the differentiation of the melanocytes as well as the aging of the subculture.10

In our material, growth was luxuriant and rapid while the subcultures were of light color but became sluggish when the cultures grew browner. Mitoses were rare in differentiated melanocytes, and less pigmented cells divided faster. Thus, Hu observed that if, instead of subculturing at the 7-10 days stage, the cultures were left for longer periods of time, the pigmented cells increased in number and size; their “activity” decreased and such large differentiated pigmented cells did not survive trypsinization.10

It is generally agreed that with the passing of time melanotic cultures lose their melanin pigment and become amelanotic.8,11 According to most observers this event is associated with acceleration of the rate of growth. Line TC491 was no exception to this general rule. The cells became amelanotic roughly nine months after initiation of this line. The time between initiation of a melanotic tissue culture and total loss of pigment varied in different reported experiments, and some tissue cultures have become amelanotic within a short time,10 whereas others were still melanotic after 20 subcultures.16 TC491 included enough melanotic cells after five months of subculturing to produce a melanotic melanoma in the heterologous host. The general morphology of cells in amelanotic and melanotic cultures has been noted to be similar in our material with the absence of pigment. Similar
observations have been reported in other melanoma lines.\textsuperscript{14} It seems that loss of pigment is an irrevocable change; when pigment had disappeared from the cells, exposure of such cultures to substances that were expected to foster melanogenesis ended negatively.\textsuperscript{15,16}

It is difficult to assess the capabilities or "potentialities" of cells with only morphological means. Cloning techniques enabled Romsdahl and Hsu to establish three stable substrains from one cell line; each substrain had a characteristic morphology and produced a characteristic amount of melanin.\textsuperscript{18} Most authors, as already stated, observed in their cultures varying numbers of fibroblasts, lymphocytes, and macrophages; some of these cells have contained melanin pigment in melanotic cultures.\textsuperscript{5,7,8,14} Some workers stated that fibroblasts were less numerous in animal than in human melanomas;\textsuperscript{15} others observed that fibroblasts did not associate with those parts of the outgrowth containing spindle-shaped melanocytes, and this was thought to be helpful in discriminating between these two cell types.\textsuperscript{16} Some investigators found that melanocytes predominated over fibroblasts and macrophages in later stages of the culturing process;\textsuperscript{8} and others have used electron microscopic techniques in differentiating between fibroblasts and melanoma cells.\textsuperscript{19} It has been stated that on morphological grounds it is often extremely difficult to discriminate between various cell types occurring in a melanoma culture, e.g. fibroblasts and bipolar melanocytes and/or between lymphocytes and round melanocytes. This statement applies even more to long-term tissue cultures lines, like TC491, when fast growing cells in crowded cultures adapted to these conditions by losing their processes and thereby exhibiting an epithelial-like morphology.

Transplantation techniques of tissue culture lines have been used in this laboratory as a tool for supplementing morphological studies and culturing techniques.\textsuperscript{17} The behavior of the human line TC491 when transferred from the test tube to a heterologous host and the behavior of specific tissue culture cells when placed into an animal will be briefly discussed. The fate of the unusual monstrous tissue culture cells mentioned previously, which were incorporating or "ingesting" melanocytes will be mentioned with specific reference to their heterotransplantation.

The morphology of the first passage of N491 was that of a well vascularized pleomorphic tumor whose cytoarchitecture had a vaguely alveolar pattern. This pattern was most evident and obvious with histological techniques demonstrating reticulum, like Laidlaw connective tissue stain. Delicate reticulum fibers were seen separating small islands of tumor cells, and at no time was a positive reticulum stain detected in the well preserved parts of the tumor; it was positive only in regions of scarring. Fascicular arrangement of the neoplastic cells, as described in variants of human melano-
nomas designated “melanosarcomas,” and in some animal melanomas, like that described in Greene,” Loustalot, et al. and others was not seen in the various passages of N491. However, the histological pattern as seen in Cloudman melanoma S91, and labelled by Loustalot, et al. as “mixed alveolar fascicular,” was encountered as a rare event, and only in some passages (see Fig. 11). The arrangement of cells in “whorled, sarcomatoid” packets, “often peripherially disposed” around venules as described by Fortner, et al. in spontaneous pigmented melanomas of hamsters was not observed in N491. The alveolar-like cytoarchitecture of N491 was consistent, and was maintained through the different passages up to the present time. The only difference between early and late passages was the melanin content of the neoplastic cells. Melanin pigment was always present in tumors that were microscopically black or brown; however, as already stated, the number of pigmented cells did not always coincide with degree of gross pigmentation and some brown tumors contained as many melanotic cells or even more than some pitch black neoplasms. The amount of melanin became minimal after the 21st passage of N491, negligible in higher passages, and not detectable after passage 57. Concomitant with the diminution of melanin was an acceleration of the rate of growth of N491. Fortner and Allen, in emphasizing the similarities between human and animal melanomas, noticed the following differences between these two species regarding the melanotic and amelanotic variants of melanomas: 1) No relationship was seen to exist between melanin content and degree of anaplasia in human tumors. The pigmented melanocarcinomas were considered to be as anaplastic and “active appearing histologically” as the amelanotic tumors, and both showed “devastating” capacity of metastasizing; 2) the pigmented melanomas of hamsters and mice were “more innocuous appearing histologically” than the amelanotic tumors. Furthermore, in mice and hamsters the capacity of the amelanotic tumors “to grow and metastasize far exceeded” that of melanotic tumors. Similar views regarding differences between melanotic and amelanotic variants in animal melanomas were expressed by other workers. The question arises whether N491 behaved like a human or an animal melanoma. Since tumor N491 never metastasized extracranially to the visceral organs, the question of metastasis in reference to pigmented and non-pigmented variants could therefore not be further evaluated in our material. This lack of metastases might be due to the fact that as a result of increased intracranial pressure the heterologous host is killed before the primary tumor can cause extracranial secondaries. N491 did have certain characteristics in common with human melanomas. Thus, no relationship existed between melanin content and magnitude of anaplasia, at least as judged by morphological means. Both melanotic and amelanotic variants were histo-
logically alike in their neoplastic populations, except that one was black and the other white. Secondary characteristics, like degree of necrosis, vascularization, lymphocytic infiltrates, stromatization were common to and similar in both variants. Yet, like the animal melanomas, it was our impression that when N491 became amelanotic the generation time of the tumor became shorter. It is possible that faster growth is coincidental and not related to the loss of melanin pigment. Nevertheless, one cannot ignore the finding that the amelanotic subline N491 Light, which developed from the 7th passage of N491, grew somewhat faster than the still melanotic parent tumor N491 (vide supra).

Comparison of N491 with other heterologously transplanted human melanomas is associated with certain inherent difficulties due to different experimental arrangements and sites of transplantation. Greene first reported the successful transplantation of a metastasis from a human "amelanotic sarcoma" of the skin to the anterior chamber of eight rabbits and four guinea pigs. Subsequent transfer of the first and only positive passage in a guinea pig to eight additional guinea pigs was unsuccessful. The morphology of the heterotransplant and the original human tumor are convincingly similar as judged by the photomicrographs. Both tumors are, in our opinion, melanocarcinomas rather than melanosarcomas. In another series of short-term experiments Greene transplanted 13 human melanomas in the anterior chamber of the guinea pig eye and seven grew. The slowest growing tumor required seven months to fill the anterior chamber. The incidence of takes and the rate of growth were without prognostic significance for the human tumor. Unfortunately the histology of the tumors was not extensively discussed. Other investigators were not successful in utilizing the anterior chamber as a site of transplantation for human uveal melanomas. Intra-embryonic and intratesticular routes, however, have been successfully utilized for the transplantation of a human melanocarcinoma. Furthermore, utilizing animal melanomas, the anterior chamber of the eye was used for the homologous and heterologous transfer with varying success.

In line TC491 monstrous giant cells were seen incorporating or phagocytizing melanocytes, which more often than not, appeared healthy and intact. Rosenberg, et al. were the first to call attention to the existence of such giant "ingesting" cells in tissue cultures of hamster melanoma. Their description and illustrations of the giant "ingesting" cells are excellent. Furthermore, these authors documented photographically the various steps of active "ingestion" of intact melanoma cells by giant cells and their subsequent disintegration within the giant cells. One of the co-authors, Kodani, it is stated, observed similar giant cells in cultures of human melanoma. Prior to this report, "phagocytosis" of melanoma cells was shown in his-
ological preparations of human and animal melanomas. Thus, Symeonides described and illustrated clearly "autophagocytosis" of melanoma cells by giant cells in a case of human amelanotic melanoma of the skin. Loustalot, et al. have also shown phagocytosis of tumor cells by other cells in Cloudman melanoma. Finally, Rosenberg, et al. described "gigantic forms with bizarre nuclei," which did not appear to be the same as those observed in culture but "resemble those described by Symeonides." "Phagocytosis" by tumor cells in general elicits diverse opinions. Willis questioned the "phagocytic powers" of tumor cells and believed that "cell inclusions" seen in tumors were "products of the cells themselves and not the remains of enclosed cells"; furthermore, when polymorphonuclear leukocytes were found within tumor cells this was thought to denote not "phagocytic ingestion" of the former by the latter, but "invasion of degenerating or unhealthy tumor cells by the leukocytes." On the other hand, Ewing believed that phagocytosis by tumor cells of "other tumor cells, even in mitotic division"..."is exhibited in great variety." The following questions arise regarding phagocytosis by melanoma cells: (1.) Are the giant ingesting cells seen in cultures tumor cells or are they a type of "wandering macrophage?" Rosenberg, et al. who first posed this question felt that the answer was undetermined. (2.) Are phagocytizing giant cells seen in culture similar to those observed in solid human and animal melanomas?

In our experiment the giant ingesting cells seen in line TC491 were similar to those seen by others in tissue cultures. Furthermore the giant ingesting cells observed in the heterologous tumor N491 were also similar to those described by others in solid melanomas. Regarding the first question, experiments undertaken in this laboratory with heterologous transplantation of tissue culture lines failed to show any growth of non-neoplastic lines; only lines arising from malignant tumors grew. Furthermore, heterologous transplantation of solid tumors in the eye or brain of guinea pigs, e.g., glioblastomas, failed to show any survival of "reactive cells" in the alien species. If the giant ingesting cells in tissue cultures were types of macrophages or types of normal reactive cells they would not have survived heterologous transfer. As to the second question posed above, one can observe that the ingesting and phagocytizing cells are not morphologically identical. Nevertheless, we do believe that the giant ingesting cells in line TC491 gave rise to giant phagocytizing cells of heterologous tumor N491. In experiments undertaken in this laboratory with transplantation of tissue cultures lines of various neoplasms, e.g., Ehrlich ascites tumor, sarcoma 180, HeLa cells etc., it was shown that primitive epithelial-like cells in culture were capable, with heterologous transfer, of producing well-differentiated neoplasms characteristic for each transplanted line. It has been postulated, therefore, that the
morphology of the primitive tissue culture cells represents an environmentally induced repression. One cannot expect the giant ingesting cells of the melanomas to look similar or identical in these two different environments, the test tube and the brain of the guinea pig. Furthermore, if the giant ingesting cells seen in the heterotransplant did not arise from the tissue culture cells, one would have to assume that they came from the host, either from stroma supplying the tumor or from reactive cells. We doubt this argument because there is no evidence of any neoplastic change in the stroma or in the macrophages of heterologous tumor N491.

SUMMARY
A tissue culture line was established from a human melanotic melanoma (TC491). The morphologic appearance of the tissue culture cells, the presence or absence of melanin pigment and the rate of growth of cells growing in monolayer fashion were assessed. These characteristics varied with the time at which the cells were examined after initiation of the line and of the subcultures. The rate of growth, evaluated by total nuclei counts, was approximately 36 hours. Nine months after initiation of the line, the cells became amelanotic.

Four months after initiation of the line, the melanotic cells were transplanted, first in the subcutaneous tissue of the conditioned hamster, and subsequently into the brain of healthy unconditioned guinea pigs. Large melanotic tumors resulted and these have been transferred from host to host up to the present time, seven and one-half years later (N491). During the 7th passage one positive animal developed an amelanotic tumor and from this an amelanotic subline was initiated (N491 Light) which grew faster than the parent melanotic tumor (N491). Up to the 21st passage the parent tumor (N491) was moderately melanotic; in subsequent passage the melanin content of the tumor cells became negligible and after the 57th passage no melanin was seen. Except for the loss of pigment no morphological differences were noted between the amelanotic and the melanotic variant of N491. The tumor is a pleomorphic melanocarcinoma with an alveolar-like cytoarchitecture. Concomitant with loss of pigment, the rate of growth of the intracerebral tumors became faster. Melanotic and amelanotic variants of heterologous tumor (N491) were compared with similar variants occurring in man and animals and the similarities and differences are discussed. No extracranial metastases occurred in N491.

Giant phagocytizing cells were observed in both the culture line TC491 and the heterologous tumor N491. These monstrous cells were compared with similar cells occurring in cultures of human and animal melanomas and in melanomas of man. Based upon our transplantation results, it was
decided that they are tumor cells showing "autophagocytosis." The morphological differences of these phagocytizing tumor cells in culture and heterotransplant were attributed to environmental influences.

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