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Suppression of the neoplastic state with the acquisition of specialized functions in cells, tissues, and organs of crown gall teratomas of tobacco

(plant tumors/reversible suppression/morphogenetic factors/nopaline)

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ABSTRACT **The neoplastic state in cells of tissues and organs that develop from cloned lines of crown gall teratomas of tobacco may be completely but reversibly suppressed. Stems and leaves found on teratoma shoots may appear morphologically normal and such organs contain all of the specialized cell types and are histologically and functionally indistinguishable from those found in normal tobacco shoots of comparable age. When, however, specialized cells of several different kinds that are present in stems and leaves of the teratomas are excised from the plant and grown on a basic culture medium they again assume their neoplastic properties. The results of this study indicate that the morphogenetic factors and mechanisms that govern so precisely growth, cellular differentiation, and organogenesis during the normal course of development can completely suppress the tumorous state, leading to the formation of cells, tissues, and organs that appear normal in every respect but are, in fact, inherently neoplastic. Whether the normal or tumor phenotype is expressed appears to depend on the activation or repression of select biosynthetic systems, one of which, the auxin system, has been identified here.**

The suppression of or a recovery from the neoplastic state resulting in a normalization of the tumor phenotype has now been demonstrated to occur in cells present in a wide spectrum of tumors that range in origin from those found in higher plant species to select tumors that occur in newts, frogs, mice, rats, and hamsters, as well as in certain cancers that develop in man. Since tumors that show these properties may be initiated by diverse physical, chemical, or biological agents, the reported results would appear to have broad biological implications. Examples of the type that best illustrate a suppression of or a recovery from the neoplastic state have recently been reviewed in detail elsewhere (1, 2). Tumor systems in which a reversible suppression of the neoplastic state can be readily achieved experimentally would appear to lend themselves admirably for a characterization of those cellular factors and regulatory mechanisms that determine phenotypic expression. It is the purpose of the present paper to describe one such tumor system found in plants in which the neoplastic state may be completely and persistently suppressed, leading to the development of cells, tissues, and organs which appear structurally, histologically, and functionally normal but which can be shown to be neoplastic when released from the morphogenetic restraints that govern so precisely the growth of all normal cells in an intact organism.

MATERIALS AND METHODS

The studies reported here were carried out with the use of a cloned line of tobacco cells (*Nicotiana tabacum* cv Havana) that had been transformed into tumor cells by the T37 strain of the crown gall bacterium *Agrobacterium tumefaciens*. This cloned line has been described briefly elsewhere (3). The basic culture medium used in these studies was that of Linsmaier and Skoog (4). Naphthalene acetic acid at a concentration of 0.5 mg/liter was used in experiments in which the basic culture medium was supplemented with an auxin. The Mn2 liquid medium of Meins (5) was used to encourage the development of tumor shoots from teratoma tissues. Pieces of teratoma tissue grown on an agar-containing basic medium and approximately 1 × 1 × 1 cm were transferred to 125-ml Erlenmeyer flasks containing 15 ml of Mn2 medium. The flasks were then placed on a reciprocal shaker in diffuse light at a temperature of 23–25°. The shoots that developed from such tissues were isolated, planted on an agar-containing basic medium, and after about a week were grafted into the region of the cambium at the cut stem ends of tobacco plants of the morphologically distinct Turkish cultivar.

Tissues derived from the grafted teratoma shoots or from normal shoots that were used for cultural studies were surface-sterilized by first washing them with Ivory soap and water. The tissues were then rinsed with water, placed for 5 min in a solution composed of Alconox 2 g, Clorox 100 ml, and water 900 ml. Following this treatment the tissues were dipped briefly in 80% ethyl alcohol and were then washed successively in three rinses of sterile distilled water.

Isolation of pigments from normal and teratoma leaves was carried out according to methods described by Kaufman *et al.* (6). Pigments present in the leaf extracts were separated and identified by thin-layer chromatography according to the methods of Egger (7).

Conversion of $^{14}CO_2$ into Sucrose. The photosynthetic abilities of normal and teratoma leaves of the same size were compared. Two 1-liter, stoppered flasks containing a small square of Whatman 3MM paper which was anchored with cellophane tape to the inside bottom edge of each flask were used. The flasks were tipped to permit the addition of 25 μCi of $Na_2{}^{14}CO_3$, after which an excess of 5 M HCl was added. The flasks were then tipped to permit the liquid to be absorbed by the paper. In each experiment, a normal tobacco leaf was placed in one flask and a teratoma leaf in the other. Care was taken to prevent the leaves from coming in contact with the HCl solution. The flasks were stoppered immediately after the leaves were added and held in a greenhouse in bright midday sunlight for 10 min. Each leaf was then removed and placed in a mortar containing sterile sea sand and boiling 80% ethanol. The leaves were thoroughly ground and the resultant brei was filtered through paper. The filtrates were flash-evaporated to dryness and redissolved in 2 ml of 80% ethanol. Aliquots containing 50 μl were chromatographed on Whatman 3MM paper, ascend-

FIG. 1. Representative responses obtained when tumor shoots of Havana tobacco origin such as those shown in (A) were implanted in the region of the cambium of the cut stem tips of normal Turkish tobacco stock plants. (B) The implanted shoot did not establish a vascular connection with the host and was forced from the main axis of the stem by the dividing tumor cells of the shoot. (C) A vascular connection was established but the stem of the implanted shoot was highly abnormal and growth was arrested. Note that the leaves appear morphologically normal. (D) Growth of the implanted shoot, although clearly abnormal, shows well-organized stem and leaves. (E) This shoot, although abnormal at the base, developed quite normally and ultimately flowered and set fertile seed.

ing, in a solvent of acetic acid, butyl acetate, and water 3:3:1. The papers were air-dried and chromatographed in a second direction, ascending, in a solvent system containing pyridine, ammonium hydroxide (28%), and isobutyl alcohol 4:2:1. The dried chromatograms were placed on x-ray film and exposed for 4 days. Identical volumes of extract were counted in an Aquasol cocktail and the teratoma leaf extract was found to contain 430×10^3 cpm, while the normal leaf extract contained 423×10^3 cpm.

Isolation and Detection of Nopaline [N^2(1,3-dicarboxypropyl)-L-arginine]. The general techniques used in this work have been described previously (8). Tissues of normal or teratoma origin to be studied were placed in liquid nitrogen, ground to powder, and lyophilized immediately. The dried samples were stored at −20° prior to use. Fifty-milligram samples of the lyophilized material were ground at 4° in a prechilled mortar with fine glass beads. The powder in each instance was placed in a centrifuge tube and 0.5 ml of cold distilled water was added. The brei was vigorously stirred for 10 sec in a vortex mixer at 10-min intervals for 1 hr. Following this treatment the material was centrifuged at $3000 \times g$ for 10 min. The clear supernatant was used immediately for analysis.

The electrophoresis apparatus used in this study consisted of two glass plates 40 × 17 cm and 0.6 cm thick and appropriate buffer wells to support the glass plates. The power source delivered a maximum of 1 kV and the electrodes were platinum. Whatman 3MM paper was cut into 57 × 17-cm strips and served as the matrix. The buffer used consisted of formic acid, acetic acid, and water 11:30:159 vol/vol.

A strip of Whatman 3MM paper was dipped in the buffer solution and blotted to remove excess buffer. It was then placed on a chilled glass plate in a 4° room, centered, and 5, 10, or 20 μl of the material to be tested was spotted 3 cm from the anode side of the plate. Authentic nopaline and arginine were dissolved in 10% acetic acid at a concentration of 2 μmol/ml. In practice the authentic materials were run between an unknown sample and the unknown containing both authentic compounds. The prepared paper strip was sandwiched between two glass plates and held firmly in place with four no. 4 Hunt clips. The ends of the paper strips dipped into the buffer wells and those parts of the paper strips extending from the surface of the buffer wells to the plates were reinforced with strips of Whatman 3MM paper to reduce evaporation at the exposed surfaces.

Electrophoresis was carried out at 1 kV for 2 hr at 4°. The paper was then air-dried in a hood.

Two electrophoretic runs were made for each unknown sample. Nopaline was detected by its characteristic position on the paper following electrophoresis, with the use of phenanthrene quinone reagent (9) and, on the second sheet, with a modified Sakaguchi reagent (10).

EXPERIMENTAL RESULTS

When cloned lines of crown gall teratoma tissue of tobacco (*N. tabacum* cv Havana) are grown on a basic culture medium they characteristically develop tumor shoots (Fig. 1A). When such shoots are isolated and grafted into the cambial region at the cut stem tip of a morphologically distinct tobacco cultivar (*N. tabacum* cv Turkish) a broad spectrum of responses may be obtained. Many of these tumor shoots failed to establish a vascular connection with the host plant and, being tumorous, developed into typical teratomatous growths, the rapidly dividing cells of which often forced what remained of the implanted shoots away from the main axis of the stem (Fig. 1B). The leaves present on the original shoot retained their characteristic morphology although they

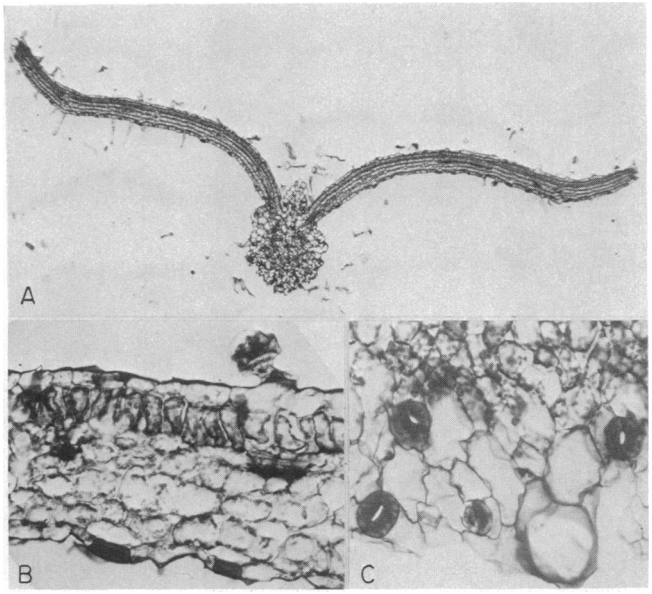

FIG. 2. Histological section of (A) a young teratoma leaf; (B) a cross section of a teratoma leaf of the type pictured in Fig. 1C. Such leaves appear histologically normal and show all of the specialized cell types found in normal Havana tobacco leaves. (C) Epidermal strip showing stomata that develop in a teratoma leaf.

often did not grow significantly in size. Other implanted teratoma shoots established a vascular connection with the host and developed quite well until they reached a height of 4–8 cm. Cessation of growth in these instances resulted from a severe disruption of the characteristic structure of the stem (Fig. 1C). The leaves found on such teratoma shoots appeared normal and retained their characteristic morphology following a breakdown of the cellular organization of the stem. A third general type of response that was observed was one in which the implanted shoot, although clearly abnormal in appearance, grew well and often showed no obvious gross disruption of the cellular organization of either most of the stem or of the leaves (Fig. 1D). Finally, some of the implanted shoots developed quite normally and some of these ultimately flowered and set fertile seed (Fig. 1E). When those seeds were sown they gave rise to Havana tobacco plants that had lost their tumorous properties and had thus recovered from the tumorous state. The present study is concerned only with teratoma shoots prior to the onset of the flowering stage.

Histological Studies of Teratoma-Derived Leaves and Stems. A histological study of leaves found to develop on teratoma shoots of the type pictured in Fig. 1C, D, and E showed that such leaves contained all of the diverse specialized cell types found in normal Havana tobacco leaves (Fig. 2A and B). The well-developed upper and lower epidermis contained typical epidermal hairs as well as glandular hairs. The stomata in the upper and lower epidermis appeared normal and showed a typical distribution on the leaf surfaces (Fig. 2B and C). There was often a well-defined palisade layer as well as underlying spongy mesophyll cells, both of which contained chloroplasts. The veins appeared histologically normal. The more normal-appearing teratoma stems also contained all of the cell types found in normal tobacco stems. When such stems were grossly abnormal in appearance the abnormality resulted largely from an excessive production of secondary xylem.

Since the leaves that developed from the teratoma shoots appeared morphologically and histologically normal, the question that arose was whether they were functionally normal as well. An analysis of the photosynthetic pigments obtained from teratoma leaves and comparable normal leaves showed that both types of leaves contained the same pigments and in essentially the same amounts. Chlorophylls a and b, α- and β-carotene, as well as neoxanthene, were identified in both normal and teratoma leaves.

Isolated teratoma and normal leaves of comparable size and age were excised from their respective plants and allowed to photosynthesize in the light in a sealed container in the presence of $^{14}CO_2$. The labeling patterns, determined by 2-dimensional paper chromatography, were essentially the same in the two types of leaves. All labeled compounds positively identified in autoradiographs of normal leaves could also be identified in teratoma leaves. These compounds included malate, citrate, glycollate/glycerate, glycine, serine, glutamine, α- and β-alanines, hexose phosphates, sugar nucleotides, and sucrose. A very few labeled compounds were detected in teratoma leaves but not in normal leaves.

The teratoma leaves appear, therefore, to be not only morphologically and histologically normal but to be functionally normal as well. These findings are subject to two possible interpretations. The first is that the specialized teratoma leaf cells are normal, resulting from the spontaneous self-healing of those cells during the development of the leaves. A second possible explanation is that those cells are inherently tumorous but that the tumorous state has been completely suppressed, leading to the development of tissues and organs that appear normal in every respect. In an attempt to distinguish between these two possibilities, fragments of leaf tissue were excised from normal leaves and from teratoma leaves and planted on a basic culture medium. The results of these studies, which are in part pictured in Fig. 3A, lower row, show that the cells at the cut edges of the leaf fragments of teratoma leaves proliferated actively and developed into typical teratomatous growths. These new growths were carried through seven successive passages at 5-week intervals on a basic culture medium without reduction in their growth rates. When normal leaf fragments were similarly isolated and planted on the basic medium a characteristic wound healing response was observed at the cut edges of the tissues without excessive overgrowths developing (Fig. 3A, upper row). Transverse and peridermal histological sections of isolated teratoma leaf tissues showed not only that the new growths developed from the vascular tissues but also all of the cells at the cut edges of the teratoma leaf fragments proliferated actively (Fig. 3B). This was evidenced further by isolating strips of lower epidermis that contained one or more layers of underlying mesophyll cells but were free of vascular tissue, and planting those strips on a basal medium. Histological studies indicated that both mesophyll and epidermal cells proliferated actively, and the resulting tissues were carried through successive passages on the basic medium with no reduction in their growth rates. Cells present in the larger teratoma leaf veins, when isolated, also proliferated autonomously on the basic culture medium. It thus appears that the teratoma leaves are composed largely if not entirely of tumor cells.

The cellular architecture of teratoma stems may appear highly disorganized or it may appear quite normal and contain all of the cell types found in normal shoots. When tissue fragments were isolated from the vascular region of the more normal-appearing teratoma stems and planted on the

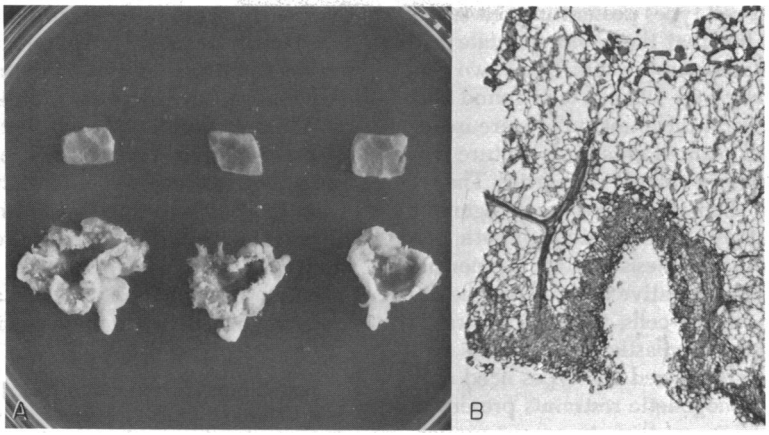

FIG. 3. (A) *Upper row,* three pieces of Havana leaf tissue isolated from three normal leaves and planted on a basic culture medium. *Lower row,* three pieces of leaf tissue isolated from three teratoma leaves of the type pictured in Fig. 1C, D, and E, and planted on the basic culture medium. Pictures were taken 16 days after the leaf tissues in both instances were planted on the basic culture medium. (B) Peridermal section of a teratoma leaf 8 days after it was excised and planted on the basic culture medium. Note that new growth arises not only from the cut end of a leaf vein but from all cells that border the cut edges of the leaf fragment.

basic culture medium the cells proliferated actively and could be carried through at least six successive transfers on that medium. Similar vascular tissue fragments isolated from normal shoots commonly produced a callus at the basal end of a tissue fragment but this new growth was of a self-limiting type. Excised teratoma pith parenchyma tissue did not grow on the basic culture medium and thus behaved in culture as did normal pith tissue. Teratoma pith fragments and normal pith fragments responded very differently, however, when planted on a basic culture medium supplemented with the synthetic auxin naphthalene acetic acid (Fig. 4). Under these conditions of culture, growth of the normal pith cells was largely if not entirely due to cell enlargement. The teratoma pith tissue fragments, on the other hand, proliferated actively. Following one passage on the auxin-containing medium, the teratoma pith cells continued to proliferate actively through seven successive passages at 5-week intervals on the basic culture medium and had thus achieved auxin autonomy. When either teratoma pith or leaf tissues that had been carried through at least three passages on the basic culture medium were grafted to cut stem tips of Turkish tobacco plants they developed into typical teratomatous growths in those instances in which the grafted tumor tissue fused with the host. These tissues were therefore tumorous.

Since the arginine analog nopaline has been reported to be a highly specific biochemical marker for crown gall teratoma tissue of tobacco (8, 11, 12), a study was undertaken to determine whether the cloned line of teratoma tissue used in these studies synthesized that compound. Since nopaline was readily detectable in that cloned line but was not found to be present in normal tobacco tissues or in habituated tobacco tissues of Havana origin, the results obtained in this study provide strong evidence that the cloned cell line used in these studies was teratoma tissue of crown gall origin. Nopaline was also readily detectable in the highly organized teratoma leaves but not in normal tobacco leaves. It was also found to be present in pith tissue isolated directly from teratoma stems as well as from actively proliferating tissues that developed from excised teratoma leaf and pith tissues grown on a basic culture medium. This could mean either that the nopaline-synthesizing system is persistently activated in even the most highly organized teratoma leaves or that nopaline, like, for example, nicotine, is synthesized elsewhere and is translocated to the leaves and stored there.

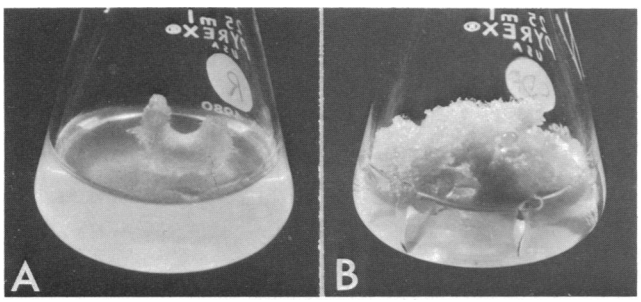

FIG. 4. Response of excised normal and teratoma pith tissue fragments planted on a basic culture medium supplemented with the synthetic auxin naphthalene acetic acid (0.5 mg/liter). (A) Normal pith. Growth results largely from cell enlargement. (B) Teratoma pith. Note active cellular proliferation occurs.

DISCUSSION AND CONCLUSIONS

When totipotential cells of tobacco origin are transformed to crown gall tumor cells by the T37 strain of the crown gall bacterium the resulting tumors (teratomas) are characterized by a capacity to organize tumor shoots. When such, often well-organized, shoots are isolated from cloned lines of teratoma tissues and grafted into appropriate hosts a wide spectrum of responses, which are described above, may be obtained. The present study is concerned with an analysis of the morphologically well-organized leaves and stems that develop from implanted teratoma shoots prior to the onset of the flowering stage in those instances in which flowering occurred. It was found that teratoma leaves appeared morphologically and histologically normal and contained all of the specialized cell types found in normal leaves of comparable age and stage of development. The teratoma leaves also contained all of the photosynthetic pigments present in normal leaves and they converted $^{14}CO_2$ into the usual spectrum of ^{14}C-labeled intermediates. All labeled intermediates positively identified in autoradiographs of normal leaves were also identified in teratoma leaves. Cells of such teratoma leaves are clearly not exhibiting neoplastic properties and they appear by all generally accepted criteria to be normal. This could mean either that a spontaneous self-healing of cells of such teratoma leaves occurred during their develop-

ment or that the specialized cell types present in such leaves were inherently neoplastic but that the tumorous state had been completely and persistently suppressed. These two possible interpretations were resolved when it was found that when specialized cells of several different types were isolated from teratoma leaves and planted on a basic culture medium they again assumed their neoplastic properties. The results of this study indicate then that those morphogenetic factors and mechanisms that govern so precisely growth, cellular differentiation, and organogenesis during the normal course of development are also operative in directing the development of teratoma shoots, the cells, tissues, and organs of which, although inherently neoplastic, may appear normal in every respect. Those specialized cell types need only to be released from the morphogenetic restraints present in an intact organism to again assume their tumorous properties.

Insight into what may occur at the cellular level during the development of a teratoma shoot appears to be provided by a study of the response of pith parenchyma cells found in teratoma stems. When such pith tissues are excised and planted on a basic culture medium, growth of the teratoma pith cells, as in the case of normal pith cells, does not occur. When, however, the teratoma pith and normal pith cells are planted on a basic culture medium supplemented with a growth substance of the auxin type, the normal and teratoma pith cells respond very differently. Under those conditions of culture growth of the normal pith cells was largely if not entirely due to cell enlargement, while the teratoma pith cells proliferated actively. It was found, further, that following one passage of teratoma pith cells on an auxin-containing medium those cells could be passaged successively on a basic culture medium without any obvious reduction in their growth rates. Not only had the teratoma pith cells again acquired auxin autonomy, but they were also found to develop into typical teratomas when grafted into appropriate hosts and, hence, were tumorous. Since it is now well established that normal pith cells require an exogenous source of both an auxin and a cell-division-promoting factor if growth accompanied by cell division is to occur, the results reported here indicate that while both the auxin and cell division factor-synthesizing systems are solidly blocked in normal pith cells, only the auxin-synthesizing system is repressed in the teratoma pith cells. This system can, moreover, again be persistently activated by forcing the teratoma pith cells into rapid growth by means of a brief exposure of those cells to an exogenous source of an auxin.

While a persistent but potentially reversible suppression of the tumorous state appears to be a characteristic feature of the vegetative phase of teratoma shoot growth, a recovery from that state leading to a normalization of the tumor cells appears to result during the reproductive phase and either it may involve the meiotic process or it may occur during fertilization or seed formation. An attempt is now being made to distinguish between these possibilities.

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