of the original population gave rise to viable colonies on normal agar plates, but 100 per cent were successful colony formers on supplemented agar plates.

The mechanism of "osmotic restoration" is obscure. It may be the result of an early injury of the cell wall synthesizing mechanism leading to a transitional imbalance between the synthesis of cell wall and protoplasmic material;‖ or, perhaps, it may be a reflection of the interference of FU with some other phase of pyrimidine metabolism.

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‡ Prolonged incubation of normal nutrient agar plates—up to 3 days—instead of the usual 16 hours did not increase the number of colonies.

§ On plates containing high concentrations of sucrose or salt the size of the colonies was appreciably smaller than on normal nutrient agar plates after identical times of incubation. When single "dwarf" colonies were restreaked on normal nutrient agar plates they gave rise to normal sized colonies and the number of such colonies on normal and on supplemented plates was the same.

‖ The role of uridine coenzymes in bacterial cell wall synthesis has been demonstrated.[10]

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*A DEMONSTRATION OF THE RECOVERY OF THE CROWN-GALL TUMOR CELL WITH THE USE OF COMPLEX TUMORS OF SINGLE-CELL ORIGIN**,†*

BY ARMIN C. BRAUN

THE ROCKEFELLER INSTITUTE

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Introduction.—It is a generally accepted belief that the cellular alteration leading to malignant tumor cell types represents a permanent change. Cells that possess low grades of neoplastic change may and frequently do become further altered in the direction of greater malignancy. Once this state has been attained, however, such cells have not been observed to turn back *en masse* toward the benign or normal states. Studies on the origin of a tumor cell (except the virus-induced tumors) have been complicated in the past by an inability to accomplish a controlled recovery of

such cells and thus permit an experimental analysis of the several hypotheses that have been advanced to account for the continued abnormal and autonomous growth of a tumor cell.

The typical crown-gall tumor cell of plants, like malignant animal cells, appears to be a permanently altered cell that reproduces true to type and against the growth of which there is no control mechanism in the host. This cell type is characterized both in the host and in culture by a capacity for continued rapid proliferation, by a limited capacity for differentiation, and by the lack of a capacity to organize tissues and organs (Fig. 1, A). Since tumor cells of this type have not in the more than 10

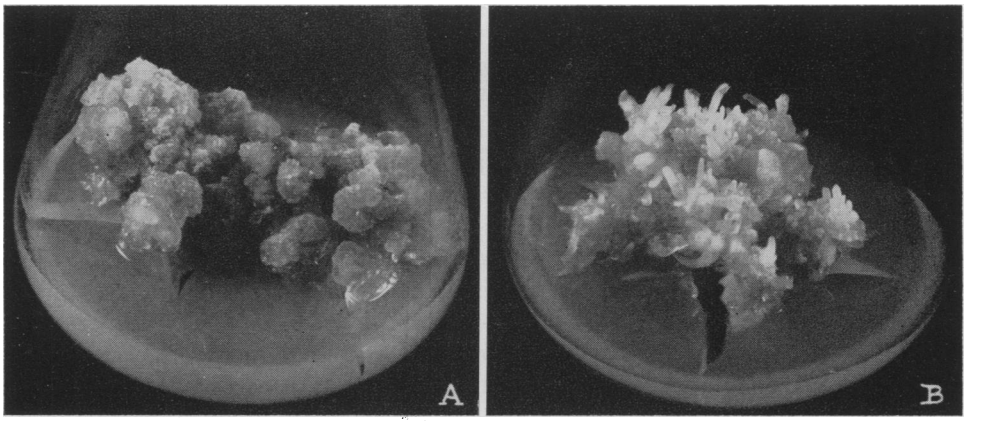

FIG. 1.—A. Typical tobacco crown-gall tumor tissue of the unorganized type. B. Crown-gall teratoma tissue of tobacco derived from a single cell. (Photographs by J. A. Carlile.)

years that they have been kept under observation shown the slightest tendency to become less autonomous, they have generally been considered to be permanently altered cells and hence unsuited for studies dealing with the recovery of a tumor cell.

A second morphologically quite distinct type of crown-gall tumor may be produced in certain plant species the cells of which possess highly developed regenerative capacities. Tumors of this type possess, when they develop in a host, and retain indefinitely when planted in culture, a pronounced capacity to organize highly abnormal leaves and buds (Fig. 1, B). The pluripotent cells of which these teratomata are composed appear to retain, despite their alteration to tumor cells, highly developed regenerative capacities.

The complex tumors, like those of the unorganized type, are transplantable. The growth pattern of the resulting tumor depends, however, upon the position that the implant occupies in a host.[1] Furthermore, cells isolated from teratomata grow indefinitely, as do cells obtained from an unorganized tumor, on a simple inorganic salts-sucrose-containing culture medium that does not support the continued growth of normal cells of the type from which the tumor cells were derived. Since the complex tumors possessed a capacity to organize abnormal tumor buds, they were found to be admirably suited and were used for studies on the origin of the crown-gall tumor cell.[2] An attempt was made in earlier investigations to distinguish between somatic mutation at the nuclear gene level and the presence of a self-perpetuating cytoplasmic entity that had assumed control of the cells and was responsible for the

continued abnormal proliferation of the affected cells. It is well known in biology that certain self-duplicating cytoplasmic entities can be eliminated from cells under conditions that favor the increased multiplication of those cells in relation to the multiplication of the self-duplicating factor. Since normal meristematic cells of a rapidly growing bud divide with far greater frequency than do most crown-gall tumor cells, it was hypothesized that, if the tumor buds found to develop from the teratomata could be forced into very rapid growth, recovery of the tumor cells might be accomplished provided the factor responsible for the continued abnormal proliferation of the tumor cells was subject to the effects of dilution in very rapidly dividing cells. The results of that study showed that when tumor shoots derived from tumor buds were forced into rapid growth by a series of graftings to healthy plants, they gradually recovered and became normal in every respect. These results suggested that the cellular alteration in crown gall did not involve a somatic mutation at the nuclear gene level since heritable changes of that type are not generally believed to be lost as a result of rapid growth. They suggested, rather, that some autonomous or partially autonomous entity, which is subject to the effects of dilution in very rapidly dividing cells, is present in and is responsible for the continuity of the tumorous properties from one cell generation to the next. This interpretation of the observed results was subsequently questioned by certain investigators because it was felt that, on the basis of the evidence presented, the possibility that the teratoma tissue was composed of a stable mixture of normal cells and tumor cells had not been eliminated.[3-5] This question could be resolved unequivocally only if clones of teratoma tissue of single-cell origin were used in the investigations. Although methods for the culture of single plant cells had not been developed at the time that the original studies were reported, such methods are now available.[6] This has permitted a re-examination of the question of the recovery of the crown-gall tumor cell. It is with that phase of the crown-gall tumor problem that the present study is concerned.

Experimental Methods.—The clone of tobacco crown-gall teratoma tissue used in this study has now been maintained in culture for somewhat more than 5 years. Throughout this period the tissue has been characterized by a pronounced capacity to organize numerous abnormal-appearing leaves and buds and to grow continuously at a moderately fast rate on a modified White's culture medium containing 1 per cent agar. The teratoma tissue was maintained in this laboratory on a modified medium containing four times the concentration of the basic salts recommended by White.[7]

The single cells used in this study were obtained as follows. The liquid culture medium consisted of White's basic medium supplemented with a 10 per cent extract of juice obtained from rapidly growing *Vinca rosea* crown-gall tumors grown in tissue culture. The tumor extract was found to favor growth of the tumor cells. This medium was further supplemented with naphthalene acetic acid at a final concentration of 0.01 mg/liter. Fifty cc of the medium was placed in each of several 250-ml Erlenmeyer flasks. The flasks were stoppered and sterilized in an autoclave.

Teratomata that had grown on a solid medium for 5–6 weeks were cut into small fragments several millimeters in diameter and a large number of such fragments were placed in the medium described above. The flasks were shaken rapidly on a re-

ciprocal shaker for a period of about 5 weeks. Microscopic examination at the end of that period showed numerous single cells and clumps of cells to be present in the medium (Fig. 2, A, B). A suspension of such cells was either transferred to fresh medium of the type described above and again shaken or it was spread evenly on an agar surface in a Petri dish for isolation of single cells.

The single cells were isolated and cultivated according to the method described by Muir, Hildebrandt, and Riker.[6] The "host" or stock tissue upon which the filter paper (Reeve Angel, crepe surface no. 202) was placed was normal *Nicotiana glutinosa* tissue that had been carried continuously in culture for 10 years on White's basic medium supplemented with 0.5 mg/liter naphthalene acetic acid and fortified with 1 per cent agar. Normal *N. glutinosa* tissue had been found to be a favorable nurse tissue for tobacco teratoma cells. In these studies a cube of the normal tissue was cut in such a way that each side had a diameter of about 7 mm. A square piece of sterile filter paper 8–9 mm in diameter was placed upon the freshly cut upper surface of the tissue fragment. The tissue containing the paper was planted

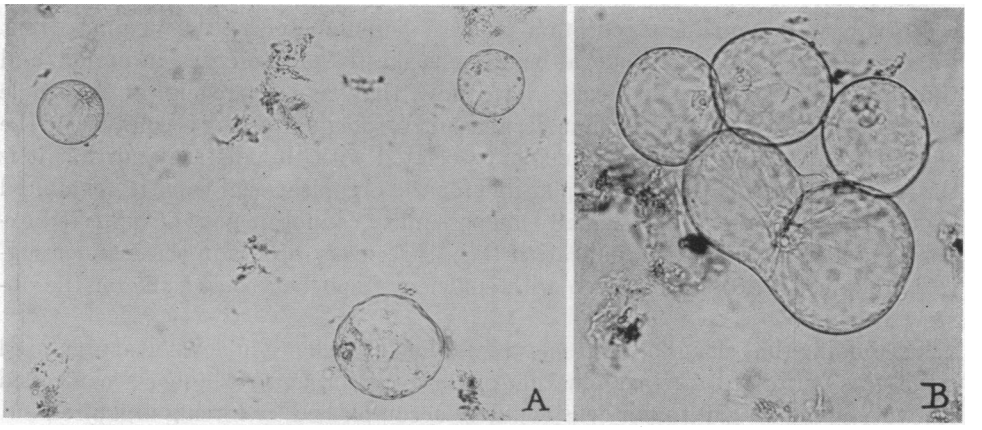

FIG. 2.—A. Single cells of the type from which the teratoma tissue shown in Fig. 1, B was derived. B. Small cluster of cells. Note that one of the cells is undergoing division. (Photographs by J. A. Carlile.)

in 125-ml Erlenmeyer flasks containing White's solid medium supplemented with naphthalene acetic acid at a concentration of 0.05 mg/liter. The tissue was allowed to remain in contact with the agar for 3 days, after which time the tissue and filter paper were removed as a unit and placed in a sterile Petri dish.

Single teratoma cells that had been planted on an agar medium in a Petri dish were isolated under a dissecting microscope with the aid of a platinum microspatula that was flattened and pointed at the tip. The single cells were planted on the filter paper. The tissue containing the filter paper was then again placed on the agar medium from which it had been removed. The entire procedure was carried out as rapidly as possible to prevent drying of the single cell.

The grafting of tissues of single-cell origin and of shoots that developed from such tissues was carried out according to methods previously described.[2,8]

Experimental Results.—Of a total of 267 single teratoma cells isolated and planted on filter paper, 12 or somewhat more than 4 per cent grew. Of these, 1 became contaminated during the course of incubation, 4 multiplied and reached diameters up to

0.3 mm but failed to continue growth, while 7 reached diameters up to 5 mm and were planted on a modified White's basic medium where they continued to grow well independently. The tissue of each of the 7 clones grew in an unorganized manner and showed no macroscopic evidence of organization at the time that they were transplanted to the basic medium from the filter paper. However, after the tissues of 6 of the 7 clones had reached diameters of between 0.7 and 1 cm, they organized morphologically abnormal structures at their surfaces. The tissue of each of the 7 clones was subdivided into 4 pieces and these were again planted on modified White's basic medium. The tissues derived from 6 of the clones grew in a manner comparable in every respect to the teratoma tissue from which the single cells were derived. Tissues of the seventh clone failed to organize during two successive passages in culture. During the third passage, morphologically highly abnormal leaves and buds developed from several of the tissues in this clone. The capacity of tissues of all clones derived from single cells to organize persisted through subsequent subdivisions and plantings on modified White's basic medium.

The results of this study indicate that the capacity of teratoma tissue of single-cell origin to organize is a reflection of the inherent potentialities of pluripotent tumor cells and is not the result, as has been suggested, of a mixture of normal and tumor cells. The possibility exists, of course, that, as the progeny of the single cells grow, some recover, perhaps as a result of unequal cell divisions, from the effects of the tumor-inducing principle associated with this disease and that it is those recovered cells that are responsible for the organizational capacity exhibited by the teratoma tissue. Although this possibility cannot at present be tested experimentally, there is every indication that, if recovery occurs in the manner suggested above, it does not happen with sufficient frequency to account for the observed results.

The finding that clones of teratoma tissue of single-cell origin developed organized structures permitted a re-examination of the question as to whether a controlled recovery of crown-gall tumor cells could be accomplished by forcing organized but morphologically abnormal tumor buds into rapid growth at the shoot apex by means of a series of graftings to healthy tobacco plants. In these studies teratoma tissues of single-cell origin and about 5–7 mm thick were grafted to cut stem ends of tobacco plants from which the axillary buds had been removed. In those instances in which the tumor grafts were successful, they developed into overgrowths of considerable size. The surfaces of these growths were covered with organized structures many of which were highly abnormal in appearance. An examination of free-hand sections of such grafted tumor tissue showed that the organized structures arose from the tumor tissue at the periphery of the expanding tumor mass and not from tissues of the normal plant upon which the graft was made.

The procedures used in an attempt to effect the recovery of crown-gall teratoma cells were similar to those previously described.[2] Shoots that developed from the tumor buds present in the teratoma grafts were removed when they were between 3 and 5 cm long and were grafted to the cut stem ends of healthy tobacco plants. Some of these shoots were very abnormal in appearance and grew slowly and very abnormally in length and in thickness. After they had reached an appropriate length, the tips were again removed and grafted to healthy tobacco plants. These commonly grew more rapidly and became more normal in appearance. The tips

of such shoots were again removed and grafted to healthy plants. They developed rapidly and appeared normal in every respect, ultimately flowering and setting seed. Such seed was sown and the resulting plants were found to be normal *Nicotiana tabacum* plants (Fig. 3). In these instances recovery appeared to have been complete. It was a gradual process that progressed in the direction of the normal as the affected shoots were forced into very rapid growth as a result of a series of graftings to healthy plants.

In certain other instances, recovery was achieved more quickly. When, for example, some of the more normal-appearing shoots that developed from the grafted teratomata of single-cell origin were grafted to the cut stem tips of healthy tobacco plants, they grew rapidly after an initial delay and a complete recovery was achieved in a single grafting. The cells of such recovered scions failed to grow on a basic culture medium that supported the continued rapid growth of crown-gall tumor cells. The cells of the recovered plants were also found to be again susceptible to transformation by the tumor-inducing principle associated with this disease.

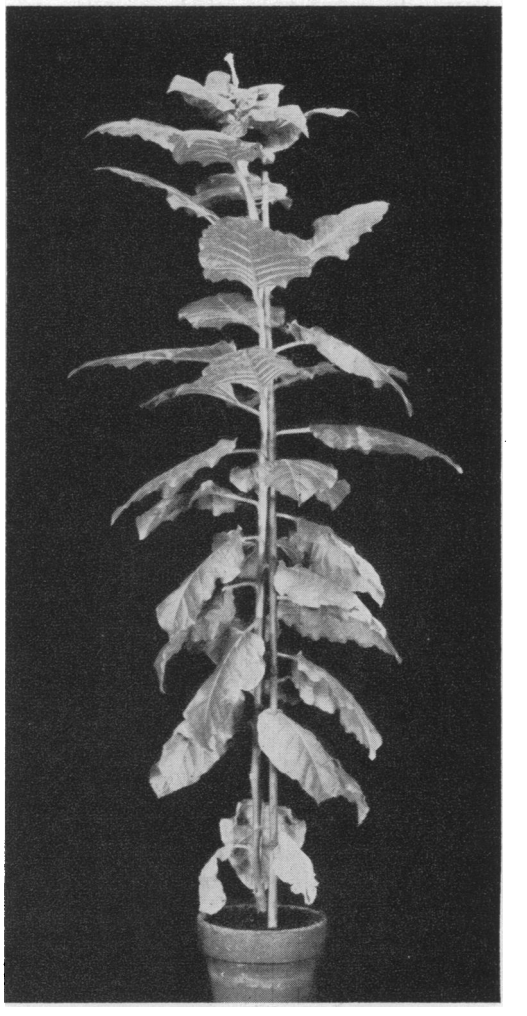

FIG. 3.—A normal tobacco plant of the type obtained by forcing morphologically abnormal shoots derived from teratoma tissue of single-cell origin into very rapid growth by means of a series of graftings to healthy plants. A complete recovery from the tumorous state has resulted from this procedure. (Photograph by J. A. Carlile.)

Discussion.—Clones of complex crown-gall tumors derived from single cells were used in investigations described above. The results of these studies indicate that when pluripotent plant cells are transformed into crown-gall tumor cells, they may retain indefinitely a capacity to organize morphologically highly abnormal leaves and buds. The organizational capacity exhibited by such cells is an expression of the inherent potentialities of the pluripotent tumor cells and does not in itself appear to affect the recovery of those cells. Only when organized structures such as those found in tumor shoots derived from the morphologically abnormal tumor buds were forced to divide very rapidly at the shoot apex, did they recover and ultimately become

normal in every respect. These findings make somatic mutation at the nuclear gene level appear unlikely as a possible explanation for the nature of the cellular alteration in crown gall. They suggest, instead, that some as yet uncharacterized cytoplasmic entity is responsible for the cellular changes that underlie the tumorous state in the crown-gall disease. This does not, of course, preclude the possibility that such postulated cytoplasmic changes may be more or less under control of the nuclear genes. These findings, which are very suggestive of some that have been encountered in microbial genetics, may be interpreted by assuming that the factor responsible for the continued abnormal growth of the crown-gall tumor cell is an autonomous or partially autonomous entity that is subject to the effects of dilution in very rapidly dividing cells.[9] While such a particle hypothesis appears to explain quite satisfactorily the experimental findings reported here, so little is in fact known about the mechanics of cell division, differentiation and organization that other interpretations, such as, for example, Delbrück's[10] suggestion involving steady state chemistry in which alternative chains of metabolic reactions are assumed to compete with one another, may apply.

This study demonstrates unequivocally, furthermore, that the progeny of a single somatic parenchymatous cell of tobacco may possess all of the potentialities necessary to reconstitute an entire tobacco plant.

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