The Activation of Two Growth-Substance Systems Accompanying the Conversion of Normal to Tumor Cells in Crown Gall

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Crown gall, a neoplastic disease of plants, is of interest because the inciting bacterium, Agrobacterium tumefaciens (Smith and Town.) Conn, converts normal plant cells to tumor cells in short periods of time. Once the cellular alteration has been accomplished, the continued abnormal proliferation of the affected host cells becomes completely independent of the inciting organism. These findings indicate that a tumor-inducing principle, which is as yet uncharacterized, passes from the bacterium to the host cells and brings about a complete and profound change in the subsequent behavior of those cells. When fragments of bacteria-free crown-gall tumor tissue (but not thoroughly ground-up tumor cells) are implanted in a healthy host of the same species, the tumor tissue fragments develop into typical crown-gall tumors.

An attempt has been made in this study to characterize within the crown-gall tumor cell the metabolic systems that are primarily affected as a result of the alteration of normal plant cells to tumor cells. Uncontrolled cellular growth in crown gall appears to be ultimately concerned with the increased availability to the tumor cell of a growth-promoting substance or substances synthesized by that cell. A recent analysis of crown-gall tumor tissue extracts (2) has shown that two separate growth factors act synergistically to promote the rapid growth by cell division of normal tobacco pith parenchyma tissue. The first of these is an auxin and is concerned, for purposes of this discussion, with cell enlargement, while the second is concerned with cell division. Neither growth factor when used by itself is effective in stimulating division of the pith cells. Auxin, however, causes individual pith cells to enlarge greatly, as was first shown by Newcomb (4). Tobacco pith cells have apparently lost, as a result of their differentiation, the capacity to produce physiologically detectable amounts of either the cell division or the cell enlargement factor. Because both growth-substance systems appeared to have been blocked in the pith cells, it was believed that experiments carried out with the use of such specialized cell types might give insight into the nature of the cellular alteration in crown gall. If, for example, the production or utilization of auxin is affected specifically as a result of the conversion of normal cells to tumor cells, then the altered pith cells should enlarge greatly without, however, dividing. If, on the other hand, the cell-division-factor synthesizing system is acted upon directly without a corresponding activation of the auxin system, then neoplastic growth should not result, because the cell division factor without auxin has been shown to be ineffective in initiating growth by cell division in tobacco pith tissue. Only if both growth-substance systems are activated simultaneously during the conversion of normal pith cells to tumor cells will a tumorous growth develop. The present paper is concerned with an attempt to test these possibilities.

MATERIALS AND METHODS

Tobacco (Nicotiana tabacum L. var. Turkish) pith tissue, which was used in this study, was isolated aseptically from the middle third of greenhouse-grown tobacco plants that were about 3 feet tall. Pith tissue fragments (1–1.3 cm. long by 0.6–0.8 cm. wide), free of internal phloem, were planted on White’s (8) basic medium containing 1 per cent agar. The virulent B6 strain of Agrobacterium tumefaciens was introduced into the tissue by means of the needle puncture method. Two avirulent strains (IIBNV6 and 5 Gly:Fe) of the crown-gall bacterium were used for comparative purposes in certain experiments.

Naphthalene acetic acid, a synthetic compound possessing auxin activity and commonly used in studies of this type, was incorporated aseptically from the middle third of greenhouse-grown tobacco plants that were about 3 feet tall. Pith tissue fragments (1–1.3 cm. long by 0.6–0.8 cm. wide), free of internal phloem, were planted on White’s (8) basic medium containing 1 per cent agar. The virulent B6 strain of Agrobacterium tumefaciens was introduced into the tissue by means of the needle puncture method. Two avirulent strains (IIBNV6 and 5 Gly:Fe) of the crown-gall bacterium were used for comparative purposes in certain experiments.

Naphthalene acetic acid, a synthetic compound possessing auxin activity and commonly used in studies of this type, was incorporated in lanolin according to the method described by Michel (3). A small drop of the slightly warmed lanolin paste with or without naphthalene acetic acid was applied in the desired position to the pith tissue. The entire procedure was carried out under sterile conditions.

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Grafting experiments were carried out to determine the effect of bacteria-free crown-gall tumor tissue on pith tissue fragments. The in vitro grafting technic used in these experiments was similar to that described by de Ropp (5). Bacteria-free crown-gall tumor tissue of tobacco was used as the scion and normal tobacco pith as the stock. The tumor tissue was nourished by placing a piece of White's basic medium containing 1 per cent agar on the upper surface of the tissue. The lower surface of the pith tissue was in contact with the agar medium present in the culture flask.

RESULTS

Response of pith tissue to the bacteria.—When tobacco pith tissue fragments were isolated, planted on White's basic medium, and inoculated with crown-gall bacteria, tumors did not develop at the inoculated surface of the tissue. Histological sections of such pith tissue showed (Fig. 1) that cell divisions resulting from wound healing did not occur at the site of inoculation or elsewhere in the tissue fragments. Earlier studies (1) dealing with the inception of the crown-gall tumor had demonstrated that cell division or the processes leading to cell division were essential if normal cells were to be altered to tumor cells. It was not unexpected, therefore, to find that tobacco pith cells were not converted to crown-gall tumor cells under the conditions of this experiment.

To ascertain whether tobacco pith cells possess the capability of being altered to crown-gall tumor cells it was necessary, as a first step, to bring about a normal wound-healing response in those cells. Vascular tissue has been shown (6) to be a source of both auxin and the cell division factor. Strands of vascular tissue were therefore isolated with the pith, planted on the basic medium, and the pith was inoculated with crown-gall bacteria. No wound-healing response and no tumor formation resulted in these experiments, as shown in Figure 2. It is known, however, that certain substances, including the two growth factors of interest in this study, are translocated basipetally in the vascular region of a plant stem. When pieces of pith, together with elements of the vascular system, were isolated and (instead of being placed on the surface of the agar medium in their normal position, as had been done in the previous experiment) were placed top-side down on the surface of the agar medium, the pith cells at their upper surface were stimulated to divide. Cell division in this instance resulted from the lateral diffusion into the pith of the two growth substances that had accumulated in the region of the vascular elements at the cut stem surface of what was originally the basal end of the tissue fragment. Inoculation of crown-gall bacteria into such stimulated pith tissue resulted in the formation of typical tumors similar to that shown in Figure 3.

Having thus found a method for inducing the division of cells in the pith, the above experiment was modified in such a way as to permit the vascular elements to remain in contact with the pith tissue only until the growth substances leading to cell division had diffused from the vascular region to the pith. This required about 3 days at 25°C. Thereafter, the vascular tissue was removed and discarded, and the pith fragments were inoculated with bacteria. Typical crown-gall tumors, such as that shown in Figure 4, were initiated in most but not in all instances.

For purposes of comparison, two avirulent strains of the crown-gall bacterium were applied separately to healing surfaces of pith tissue fragments treated as described above. Both strains appeared to induce callus formation somewhat in excess of that found in comparable but uninoculated pith tissue fragments. This finding can probably be accounted for by auxin produced by the avirulent strains. The callus tissue found to develop on pith inoculated with the nonpathogenic bacteria was greenish in color, as was that present in the uninoculated control tissue. Tumors induced in such pith tissue by the virulent strain were, on the other hand, glistening white in color. The amount of new growth that developed subsequent to inoculation with virulent bacteria was far greater than that found in the other two instances.

Response of pith tissue to naphthalene acetic acid.—Since auxin is one of the two growth factors being considered in this study, it was of interest to compare the results obtained in the previous section with the growth responses of comparable pith tissue treated with a substance possessing auxin activity. Naphthalene acetic acid (NAA), which was used in this investigation, was incorporated in lanolin and applied to isolated pith tissue fragments. Concentrations of 0.1 mg. and 0.33 mg NAA/gm of lanolin were found to be suitable and were used in most experiments.

Freshly isolated pith without vascular elements, and having what was originally the basal end of the fragment placed up and away from the surface of the agar medium, responded only with cell enlargement at the treated surface to the two concentrations of naphthalene acetic acid used. The affected cells were held together loosely and projected in all directions from the tissue mass (Fig. 5). Cell division was, as far as could be determined, absent, indicating lack of the cell division factor in the freshly isolated pith tissue. When, on the
other hand, vascular elements were permitted to remain in contact with the pith for 3 days before being removed and discarded, the pith cells commonly responded to application of the naphthalene acetic acid with growth by cell division (Fig. 6). Growth, in this instance, although limited, bore a superficial resemblance to crown-gall tumor tissue. Higher concentrations (50–100 mg NAA/gm lanolin) of naphthalene acetic acid applied to pith tissues of this type caused a pronounced auxin response involving mostly enlargement with only limited division of the pith cells at the treated surface.

Response of pith cells to tumor tissue transplants. —Although the second growth factor, which acts in association with an auxin and which is concerned with cell division, has been demonstrated (2, 7) to be present in extracts of certain crown-gall tumor tissue, it could not be determined unequivocally from previous experiments whether this substance was an artifact produced in the extracts following maceration of the tissue or whether it was actively synthesized by tumor cells during the growth process. To study this question, bacteria-free crown-gall tumor tissue of tobacco was grafted to normal tobacco pith tissue. The tumor tissue in one type of experiment was placed in intimate contact with what was originally the basal surface of freshly isolated pith tissue. Since cell divisions did not occur in such pith tissue fragments, a successful graft union was not accomplished between tumor and pith cells. In these experiments the tumor tissue had no demonstrable stimulatory effect on the underlying pith tissue, despite the fact that the former grew appreciably. The line of demarcation between tumor tissue and pith was very sharp, as seen in Figure 7.

When, on the other hand, vascular tissue was isolated with pith and was allowed to remain in contact with the pith cells for 3 days before being removed and discarded, active cell division occurred at what was originally the basal surface of the pith fragment. Tobacco crown-gall tumor tissue placed in contact with that surface formed a successful graft union. A pronounced stimulation of cells was observed within a 3-week period. As a result of growth by cell division of the stimulated cells, the tumor fragment was raised considerably above the original surface of the pith tissue fragment, as shown in Figure 8. The cells that were stimulated to divide as a result of their contact with tumor tissue were white, or in some instances greenish in color, as was the original pith tissue fragment. The tobacco tumor tissue scion, on the other hand, was brown in color, showing that the stimulated cells between the tumor tissue and the pith tissue fragment were derived from pith cells. Histological studies suggested that this interpretation was probably correct. The division of a large pith cell into as many as ten smaller cells that were clearly confined within the wall of the original pith cell was noted when histological examinations of the tissues were made soon after the graft union had succeeded. That the stimulated cells were not tumor cells was demonstrated, furthermore, by their inability to grow appreciably when planted on White's basic medium. Crown-gall tumor tissue of tobacco is autonomous for the two growth factors and develops readily on that medium. Because of the unusually vigorous response of the pith, the results obtained in this experiment have been interpreted to indicate that both growth substances are synthesized by the tumor tissue during growth. They were demonstrable in vivo in this test system only when a graft union occurred between pith stock and tumor scion. The reason for this is not yet clear, since earlier studies showed that both substances diffuse readily from an agar medium into pith cells.

DISCUSSION

The results of this study indicate that, as a consequence of the alteration of normal tobacco pith cells to tumor cells, the affected cells achieve autonomy in two essential directions. Prior to their alteration to tumor cells the normal pith cells did not produce physiologically detectable amounts of either the growth factor concerned with cell enlargement or that concerned with cell division, while, following their conversion to tumor cells, both substances were produced. If both growth-substance synthesizing systems had not been activated following cellular transformation, growth by cell division and hence tumor formation would not have resulted in the test system used in these experiments. It nevertheless seems unlikely that the tumor-inducing principle in crown gall, which appears to be highly specific in its action, acts directly on the two distinct biosynthetic pathways simultaneously by accomplishing either the removal of two normally occurring inhibitory systems or by effecting an increased synthesis or a more efficient utilization of both growth substances simultaneously but independently by the tumor cell. In the light of present knowledge, a more likely explanation would appear to be that this principle exerts its specific effect on one of the growth-substance systems, resulting in the production of greater than regulatory amounts of that substance by the tumor cell. Concomitant with this alteration, a change is effected in the second system, as a result of which the cell becomes autonomous for both growth factors.
While normal tobacco pith cells require an externally supplied source of both an auxin and a cell division factor for growth by cell division, certain other plant cell types need only the addition of an auxin to the basic medium for their continued growth in culture. Such isolated cell types appear capable of synthesizing the cell division factor. They respond readily to wounding with cell division. In these instances, in contrast with normal pith cells, the cell-division-factor synthesizing mechanism appears to be very lightly blocked or not blocked at all. Such cell types are readily converted to tumor cells by crown-gall bacteria. This suggests that the auxin mechanism is affected in some as yet unknown manner by the tumor-inducing principle, leading to an increased synthesis or decreased destruction of auxin by the cell. Since cell-division-factor synthesis is normally functional in cells of this type, the presence of greater than regulatory amounts of auxin in the cells permits the continued unregulated growth of those cells. Earlier studies (1) have demonstrated, and the present study has confirmed, that cell division or the processes leading to cell division are essential if normal plant cells are to be transformed to tumor cells in crown gall. The normal wound-healing response may thus serve a dual function in the transformation process: (a) to make vulnerable to alteration the cellular system specifically affected by the tumor-inducing principle, and (b) to set into operation the normal processes of cell division which, in the presence of the unregulated production of one of the growth factors, continue to be functional indefinitely.

Fig. 1.—Longitudinal section of tobacco pith tissue. The tissue fragment was planted on the basic culture medium with its physiological base away from the agar surface. Three days after isolation the tissue was inoculated with virulent bacteria. The tissue was fixed and sectioned 6 weeks after inoculation. Note absence of wound healing; no tumor developed on the tissue fragment.

Fig. 2.—Longitudinal section of tobacco pith tissue containing strands of vascular tissue (at left of section). The stem fragment was planted on the agar medium in its normal position with the physiological top away from the agar surface. The tissue was inoculated in the pith 3 days after isolation. The tissue was fixed 6 weeks after inoculation. Neither wound healing nor tumor formation resulted at the point of inoculation.

Fig. 3.—Same as Figure 2, except that the tissue fragment was placed in an inverted position so that the physiological base was away from the agar surface. Normal wound healing occurred, and a typical tumor developed at the point of inoculation.

Fig. 4.—Same as Figure 3, except that vascular tissue was permitted to remain in contact with the pith tissue for only 3 days after isolation. Thereafter the vascular elements were removed and discarded, while the pith tissue was inoculated with bacteria. Normal wound healing occurred. A typical tumor developed at the point of inoculation. Compare with Figure 1.

Fig. 5.—Same as Figure 1, except that pith tissue was treated with naphthalene acetic acid (0.33 mg/gm lanolin) instead of being inoculated with bacteria. Note cells at upper treated surface of the tissue have enlarged but have not divided, indicating that the cell division factor was absent in the pith.

Fig. 6.—Same as Figure 4, except that pith tissue that had been in contact with vascular elements for only 3 days was treated with naphthalene acetic acid (0.33 mg/gm lanolin) instead of being inoculated with bacteria. New growth by cell division has occurred, indicating the presence of sufficient cell-division factor in the pith to permit limited stimulation.

Fig. 7.—Bacteria-free crown-gall tumor tissue of tobacco planted on the physiological base of tobacco pith tissue that had not been in contact with vascular tissue following inoculation. Note line of demarcation between normal and tumor tissue is sharp. Since the pith cells did not divide, neither graft union nor stimulation of those cells occurred. The tissues were fixed and sectioned 2 months after isolation.

Fig. 8.—Bacteria-free crown-gall tumor tissue of tobacco planted on the physiological base of tobacco pith tissue. The pith had been in contact with vascular tissue for 3 days following inoculation. After 3 days the vascular elements were removed and discarded, and the tumor was placed on the healing pith surface. A true graft union occurred between tumor and normal tissue, resulting in a very considerable stimulation of pith cells. Note that as a result of growth of the pith cells the tumor tissue has been raised above the surface of the pith fragment. The tissue was fixed and sectioned 3 weeks after the graft had been made.

**SUMMARY**

An essential, if perhaps not the essential, difference between the normal tobacco pith cell and the crown-gall tumor cell appears to be concerned at a physiological level with the unmasking of two growth-substance synthesizing systems, as a result of which the transition from normal cell to tumor cell is accomplished. These systems are subject to very rigid control in normal pith cells.

**REFERENCES**


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