TUMOR FORMATION BY ATTENUATED CROWN-GALL BACTERIA IN THE PRESENCE OF GROWTH-PROMOTING SUBSTANCES

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It has recently been shown[1, 2] that crown-gall tumor cells, when freed of the original inciting agent, *Phytomonas tumefaciens* (Smith and Town.), Bergey, *et al.*, are capable of autonomous development. The fact that these tumor cells when grown *in vitro* retain indefinitely their peculiar cultural and cytological characteristics as well as their tumor-inducing capacity in the absence of any demonstrable stimulating agent indicates that the host cells when acted upon by *P. tumefaciens* become permanently altered. They behave like malignant animal cells in that they (1) show uncontrolled growth in the initial host, (2) can be transplanted successfully to hosts of the same species from which they were derived or to closely related hosts and there continue their uncontrolled growth and (3) retain indefinitely their ability to produce tumors and to grow autonomously *in vivo*. The nature of the fundamental change which occurs in the cells is at present not known. However, a better understanding of the factor or combination of factors involved in tumor formation should be of aid in the clarification of the physiological basis for the development of this neoplastic growth.

In recent years a number of investigators have attempted to demonstrate a possible relationship between growth-stimulating substances and tumor development. Certain of these workers[3, 4, 5, 6] have drawn a parallel between the reaction of plant tissues treated with β-indole acetic acid, a substance produced by the crown-gall organism from tryptophane, and the reaction of similar plant material inoculated with a living culture of *Phytomonas tumefaciens*. Although Riker and his associates[7] have confirmed the experimental results of the above workers, they deny the importance of β-indole acetic acid or similar growth substances in tumor formation and conclude that, as far as they are aware, *P. tumefaciens* is pathogenic independently of these materials. Levine[8] agrees with Riker and states that no evidence has thus far been adduced to show that the mechanism of tumor formation is due to the presence of β-indole acetic acid.

The earlier work of Locke, Riker and Duggar[9] indicated, nevertheless, that there was a high level of growth-promoting substance present in plants inoculated with a virulent culture of the crown-gall organism. This was evidenced by marked growth responses such as epinasty of the leaf petioles, inhibition of the axillary buds, etc., that resulted, in addition

to tumor formation, in certain host plants. Plants inoculated with an attenuated culture show these growth responses to a much lesser degree or not at all. This would suggest that growth substances may be a limiting factor in tumor formation by the attenuated culture. Locke, *et al.*, were not able, however, to bring about an appreciable increase in the size of the tumors formed by supplementing the attenuated culture with β-indole acetic acid.

It is the purpose of the present paper to demonstrate that an attenuated culture of the crown-gall organism, when supplemented with certain growth-promoting substances, is capable of inducing the formation of large tumors in tomato plants. Evidence is presented, furthermore, that tissue fragments from such experimentally induced tumors retain undiminished their tumor-inducing capacity upon transplantation.

Methods.—The attenuated isolate of *Phytomonas tumefaciens* used in these experiments was first described by Hendrickson, *et al.*,[10] and designated by them as the *A*66 strain. This isolate is capable of inducing cellular proliferation at the point of inoculation somewhat in excess of the normal wound response of tomato plants, but it is unable to cause the development of crown-gall tumors under ordinary conditions (Fig. 1 (*b*)). Available for comparative purposes was the *A*6 strain from which the attenuated *A*66 culture was derived. The *A*6 strain is highly virulent, as shown by its ability to incite the formation of massive galls at points of inoculation (Fig. 1 (*a*)).

The host plant used throughout the tests was the tomato (*Lycopersicum esculentum* Mill. var. Bonny Best). Young seedlings were transplanted to 4-inch pots and kept on a greenhouse bench throughout the course of an experiment. When the plants reached a height of approximately 15 inches, they were decapitated and inoculated by means of the needle-puncture method with the attenuated *A*66 culture. Two inoculations were made in each plant at distances of approximately $^1/_4$ and $^3/_4$ inch below the decapitated stem. Four days after inoculation, chemically pure growth substances in concentrations of 0.5, 1.0, 1.5 and 2.0% in lanolin were applied separately to the decapitated stumps of the inoculated plants. At intervals of 7 days, the growth substances were renewed until 3 applications had been made. The growth substances used included α-naphthalene acetic acid, β-indole acetic acid and γ-indole butyric acid. Controls for the above experiments were set up as follows: One series of decapitated plants was punctured with a sterile needle instead of being inoculated with the attenuated culture but received the corresponding growth-substance applications; in a second series, pure lanolin was applied to plants inoculated with the attenuated culture.

The following technique, successfully employed by us in the tissue transplantation experiments to be described later, was adopted after a number

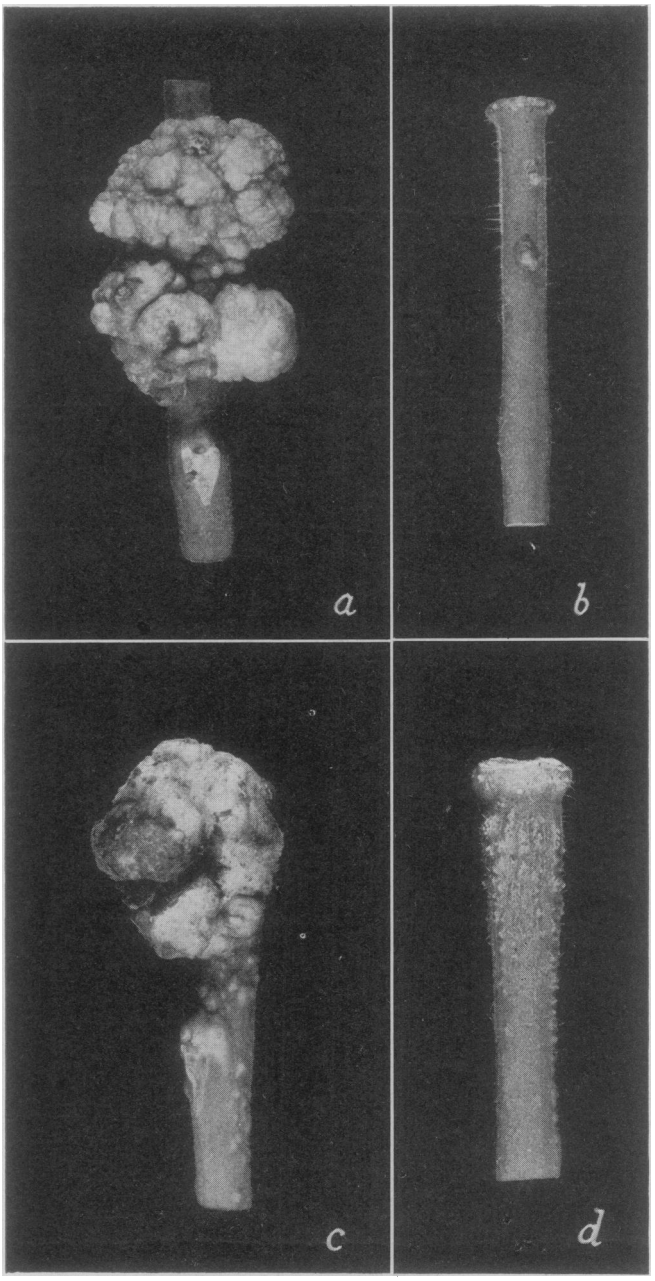

FIGURE 1

Decapitated tomato plants inoculated with (*a*) the virulent *A*6 culture, (*b*) the attenuated *A*66 culture, (*c*) the attenuated *A*66 culture and treated with synthetic growth-promoting substance. (*d*) Uninoculated, punctured with a sterile needle and treated with synthetic growth-promoting substance. Note similarity of tumors formed in (*a*) and (*c*). (Photographs by J. A. Carlile.)

of preliminary experiments established its superiority over the common grafting procedures. With the aid of a sharp scalpel, two parallel incisions were made along the axis of the stem, usually well above the middle of the plant. The incisions were approximately $^1/_4$ inch apart and 1 inch in length. By careful insertion of the scalpel blade between the two incisions, the epidermal strip, together with the subjacent cortical and phloem elements, was pried loose from the main body of the stem and the desired tumor tissue fragment was then placed in the space between the flap and the stem. The implanted tissue was wrapped securely with "Sterilastic" tape which was removed 12 to 14 days later. The successful development of the implant appeared to be dependent upon early and satisfactory vascularization between the host and the tissue fragment. Tissues inserted inside or outside the confines of the vascular ring failed to develop in the majority of cases.

Experimental Results.—The three growth-promoting substances used, α-naphthalene acetic acid, γ-indole butyric acid and β-indole acetic acid were all found to have a pronounced stimulating effect causing the development of large tumors in plants that had been previously inoculated with the attenuated *A*66 culture. However, consistent differences in the degree of stimulation, rate of tumor development and morphology of the resulting tumor mass were exhibited by the growth-promoting substances. The stimulating action of γ-indole butyric acid was usually observed somewhat earlier than that of α-naphthalene acetic acid. The latter, however, gave rise to tumors that most closely resembled those initiated and stimulated by the virulent *A*6 culture. These tumors were white in color and irregular in contour, while those produced with the aid of γ-indole butyric acid were regular in contour and had a decidedly greenish tinge. In most instances the tumors stimulated by these two substances grew rapidly and became very large. Sizable tumors frequently developed after 3 applications of 0.5% of these growth substances in lanolin or following a single application of 1.0%. β-Indole acetic acid was not as effective in our hands as were the other two materials. Higher concentrations, usually 3 applications of 2.0% in lanolin, were required to bring about a tumor stimulation comparable to that secured with lower concentrations of the other two substances. The β-indole acetic acid-stimulated tumors, however, frequently grew to be very large and resembled closely those stimulated by γ-indole butyric acid. Figures 1 (*c*), 2 (*a*) and 2 (*b*) show, respectively, the stimulatory effects of 3 applications of 1.0% of α-naphthalene acetic acid, 2.0% of β-indole acetic acid and 1.0% of γ-indole butyric acid in lanolin. The control, an attenuated culture-inoculated plant treated with pure lanolin, is shown in figure 1 (*d*).

The stimulatory effects of these growth substances on tumor development by the attenuated culture were not always evident even though

conditions were favorable. Thus, under completely comparable conditions, tumors on some plants would be stimulated to further development while not on others. The explanation of this variability may involve differences in rate of diffusion of growth-promoting substances in different plants.

The action of α-naphthalene acetic acid, β-indole acetic acid and γ-indole butyric acid on tomato plants has been studied by many investigators in recent years. In our work as well as in the work of others it has been found that these substances in the concentrations commonly used are

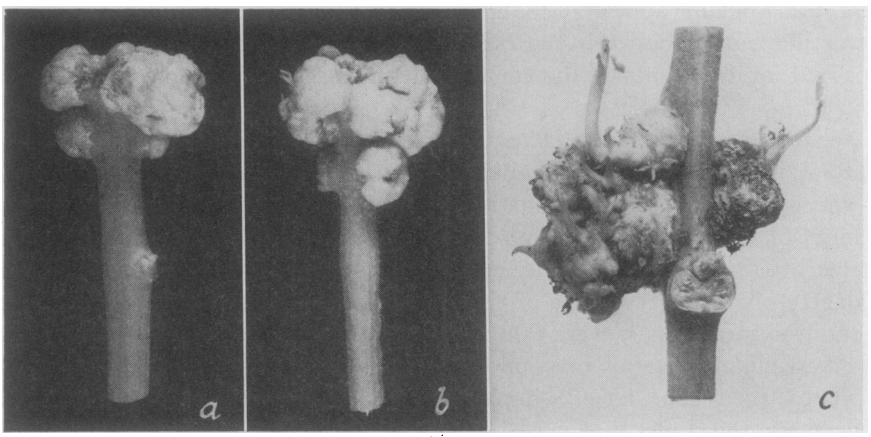

FIGURE 2

Decapitated tomato plants inoculated with the attenuated *A*66 culture and stimulated with (*a*) β-indole acetic acid, (*b*) γ-indole butyric acid and (*c*) the plant growth hormones. (Photographs by J. A. Carlile.)

essentially root stimulants, although some hypertrophied and hyperplastic tissues are formed. The latter present a histological picture not unlike that of crown-gall tumor tissue. The hypertrophied and hyperplastic cells are, however, endowed with very limited powers of proliferation and continue to develop only as long as they are kept stimulated by the growth substances. We have never observed overgrowths to be produced on tomato plants even by repeated applications of these substances that are comparable either in size or rate of development to those produced by the crown-gall organism. Figure 1 (*a*) shows the typical reaction of a decapitated tomato stem which had received 3 applications of 1.0% of γ-indole butyric acid in lanolin. A limited amount of cellular proliferation has occurred at the cut surface where the growth substance was applied; below the cut surface there are found numerous root primordia which under humid conditions would develop into true roots. The action of

α-naphthalene acetic acid and β-indole acetic acid on decapitated tomato stems is very similar.

The discovery that synthetic growth substances were able to stimulate the development of tumors by the attenuated culture strengthened our previous belief regarding the probable rôle of the host growth hormones in the development of these neoplastic growths. It had been observed that the removal of axillary shoots from decapitated tomato plants inoculated with the attenuated *A*66 culture frequently resulted in forcing the development of small adventitious buds on or in the proximity of the proliferating tissue. The further expansion and development of these buds were often accompanied by an expansion in the mass of the subjacent tumor tissue which increased at a tremendous rate and finally reached extremely large dimensions (Fig. 2 (*c*)). Because expanding buds are known to be a rich source of growth substances, it appears likely that the plant hormones secreted by the buds served as the stimulating agent in much the same manner as did the synthetic growth-promoting materials described above. No such stimulation was observed in those cases where the adventitious buds failed to develop. There is little question but that these "leafy galls" are composed largely of tumor tissue. Our evidence for this claim will be advanced in a later section.

The possibility that the application of the growth substances had significantly altered the degree of virulence of the attenuated culture was also investigated. Bacterial isolations were made from many of the artificially stimulated tumors by means of the usual poured-plate procedures. Large numbers of the bacterial colonies that developed were reinoculated into healthy tomato plants. In addition, tumor tissue fragments were thoroughly ground up with a small amount of nutrient dextrose broth in a mortar and the resulting suspension inoculated directly into healthy tomato plants. The results from many such tests demonstrated conclusively that the attenuated culture had not increased in virulence. It still produced only slight proliferation comparable to that produced by the stock *A*66 culture. The conclusion seems justified, therefore, that the primary effect of the growth substances was to stimulate the host cells rather than to bring about a change in the virulence of the bacteria themselves. Additional evidence in support of this conclusion is presented below.

Transplantability of Tumor Tissue.—Perhaps the most distinctive characteristic of malignant cells is their relative independence of the conditions which govern the growth of normal tissues. All normal cells are subject to a rigid control mechanism when growing *in vivo*, and it is only transformed cells such as are found in neoplasms that react differently. These abnormal cells fail to respond to the morphogenetic restraints which direct the growth of cells in a healthy organism. As was pointed out earlier,

crown-gall tumor cells, like most malignant animal cells, appear to be of a type which is capable of unlimited transplantability and tumor-inducing capacity under favorable conditions. We have used, therefore, these criteria in determining whether the attenuated *A*66 culture of *Phytomonas tumefaciens* is capable, as is the virulent culture, of permanently altering the host cells.

Fragments from tumors initiated by the attentuated culture and stimulated with α-naphthalene acetic acid, γ-indole butyric acid and the natural plant growth hormones were implanted under the epidermis of tomato plants. It was soon observed that in those cases where a vascular connection with the host was clearly established the tissue fragments developed into typical crown-gall tumors in relatively short periods of time. This was especially true of the α-naphthalene acetic acid and γ-indole butyric acid-stimulated tissue which in many instances developed into large tumors 3 to 4 cm. in diameter within a period of 4 to 5 weeks. On the other hand, the tissue fragments from the plant growth hormone-stimulated "leafy" galls developed more slowly and usually did not exceed 1.5 to 2 cm. diameter in 5 weeks of development.

As indicated above, the gross morphological characteristics of the tumors initiated by the attenuated culture and stimulated by various growth substances differed considerably. However, upon transplantation of the various tumors, all of the resulting overgrowths appeared similar and could not be distinguished from each other except possibly by their respective rates of development. The tumors were for the most part a very light brown in color and of a fairly regular contour.

We have now successfully carried some of these tumors through 5 successive passages in tomato plants over a period of more than 6 months. These tumors have continued to develop at an undiminished rate throughout this period. Figure 3 (*a*) shows a typical third-generation tumor transplant after 4 weeks of development.

Additional bacterial isolations were made from these transplanted tumors to determine whether the degree of virulence of the attenuated culture had been in any way altered during the course of the experiments. It was found after testing several hundred isolates on tomato plants that no increase in virulence over that of the stock *A*66 culture had occurred. It seemed logical to assume, therefore, that the mere presence of these attenuated organisms in the tissues could not account for the rapid expansion of the transplanted fragments and that it must be the host cells themselves that had been altered to such a degree that they were now developing autonomously. This assumption was definitely established by our subsequent discovery that certain tissue fragments of the γ-indole butyric acid-stimulated tumors were entirely free of *Phytomonas tumefaciens*. Whether the bacteria had died out in these fragments or whether

the rapidly developing tumor tissue had grown away from the bacteria is not known. The bacteria-free tissues have been carried through 3 transplant generations involving a period of more than 3 months. All attempts to demonstrate the presence of the crown-gall bacteria in these

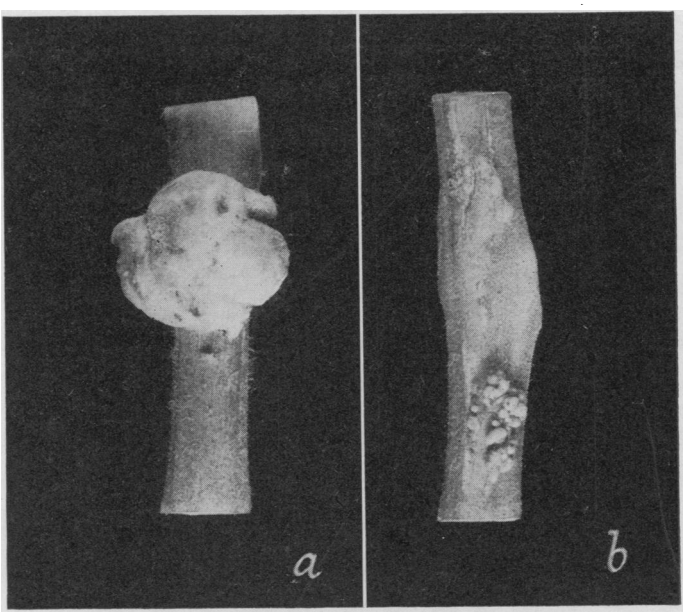

FIGURE 3

(*a*) Typical third-generation implant (originally derived from an experimentally induced tumor, Fig. 1 (*c*)) after 4 weeks of development. (*b*) Typical first-generation implants of tissue stimulated with growth-promoting substance (see Fig. 1 (*d*)) after 6 weeks of development. Two general types of response are illustrated. In the upper implant the tissue remained smooth and made but slight growth. The nodular root-like protuberances and limited growth of the lower implant characterized the second type of response. (Photographs by J. A. Carlile.)

tissues by the usual isolation procedures have failed. The bacteria-free tumor tissue fragments grew rapidly and developed into large neoplastic growths indistinguishable morphologically from the bacteria-containing tumors. On the basis of these results, it can be stated, therefore, that the attenuated bacteria are capable of altering the host cells to tumor cells which after stimulation are apparently capable of autonomous development.

The controls for the experiments described in the preceding pages were uninoculated decapitated tomato plants treated with 1.0, 1.5 and 2.0%

of β-indole acetic acid and α-naphthalene acetic acid in lanolin, as explained earlier. These materials were found to be essentially root-forming substances, although they did cause a limited amount of cellular proliferation. Fragments of these growth substance-stimulated tissues were grafted into tomato plants in the same manner as were the tumor tissue fragments. Similar grafting experiments were carried out on three separate occasions with the object of determining whether the growth substances used here were capable of permanently altering the host cells. In no instance did we find evidence suggesting that such a change had occurred. The tissue implants seldom increased to more than twice their original size and never developed into tumors. Two general types of responses were observed (Fig. 3 (*b*)). In one type of response the tissue implant gave rise to numerous nodular root-like protuberances which seldom developed into true roots, although occasionally roots 1 to 2 cm. in length were observed. The second type could be likened to a slow healing response, the inserted tissue remaining smooth and making but slight growth. It is apparent that the host cells, when acted upon by these growth substances alone, are incapable even after successful transplantation of inducing the formation of tumors. It is our belief that the growth substances used in this study served merely to stimulate cells previously altered by the attenuated culture.

Discussion and Conclusions.—It has been demonstrated that an attenuated culture of *Phytomonas tumefaciens* is capable of forming large tumors in tomato plants when supplemented with the plant growth hormones or synthetic growth-promoting substances. This growth substance reaction does not appear to be specific inasmuch as any one of several different growth substances, including that produced by the host plant, may serve as the stimulating agent. Furthermore, the gross morphological appearance of the tumors formed when an attenuated culture is supplemented with a growth substance varies with the substance used. Of such tumors, those stimulated by α-naphthalene acetic acid approximate most closely those induced by the virulent culture on tomato in that they are white in color and irregular in contour.

It has further been demonstrated by means of grafting experiments that the cells of these experimentally induced tumors can be transplanted in series and subsequently develop into large tumors which may reach a size of 3 to 4 cm. in a period of 5 weeks. Since certain of these tumors are apparently bacteria-free and since it has been shown in the case of those tumors which are not bacteria-free that the degree of virulence of the attenuated culture itself has not been altered by the application of growth substances, it is concluded that the attenuated culture like the virulent culture is capable of altering the host cells to tumor cells. However, the attenuated culture alone is unable to stimulate the altered cells to any

appreciable extent. When artificially stimulated with growth substances, these altered cells are apparently capable, as are those stimulated by the virulent culture, of uncontrolled growth *in vivo*. Thus, there appear to be at least two distinct phases involved in tumor formation. In the first phase, the normal host cells are changed to tumor cells which without stimulation will not develop into a neoplastic growth. The second phase consists in the stimulation of the changed cells to continued multiplication by a growth substance, resulting ultimately in the formation of a tumor.

The question as to whether a single substance or two or more substances are involved in the alteration and stimulation of the tumor cells remains as yet unanswered. If only a single substance is involved, however, it must differ from the growth substances used here because the cells of the tomato plant stimulated by these substances alone do not, upon transplantation, induce the formation of tumors. If two or more substances are involved, then one of the common growth-promoting substances might conceivably act as the stimulating agent. The difference between the virulent and the attenuated cultures may lie in the relative amounts of growth substances produced by each, either as a product of bacterial metabolism or by the host under the influence of these organisms. The fact that pronounced growth substance responses are characteristically shown by tomato plants inoculated with the virulent culture and are absent in similar plants inoculated with the attenuated culture lends credence to this belief.

Once the altered host cells are sufficiently stimulated, they are apparently capable of indefinite multiplication and tumor formation under favorable conditions without the additional application of growth-promoting substances. The altered cells then continue to multiply autonomously and retain, perhaps indefinitely, their tumor-inducing capacity.

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