Mechanisms of Tumor Induction in Crown Gall
I. Production and Pathogenicity of Spheroplasts of Agrobacterium tumefaciens

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Summary

The hypothesis that crown gall tumorigenesis requires the formation of spheroplasts was tested by comparing the tumor-inducing ability of populations of both permanent and revertible spheroplasts of Agrobacterium tumefaciens with that of populations of the typical rod form of the bacterium. Permanent spheroplasts produced by serial transfers in medium containing 0.005 M D-methionine did not produce tumors in either Kalanchoe or Boston daisy. The efficiency of tumor induction in Kalanchoe stems was lower for glycine-induced revertable spheroplasts than for rod forms. In addition, a progressive decrease in tumor-inducing ability, detectable within 30 min after the addition of glycine to bacterial cultures, was also demonstrated in primary pinto-bean leaves by means of the quantitative assay of for rod forms. In addition, a progressive decrease in tumor-inducing ability, detectable within 30 min after the addition of glycine to bacterial cultures, was also demonstrated in primary pinto-bean leaves by means of the quantitative assay of Lippincott and Heberlein. A study of attenuation among glycine-resistant clones of the crown gall bacterium revealed that the capacity for tumor induction is not lost simultaneously for all hosts. It was also observed that attenuated, glycine-resistant mutants may regain the capacity for tumor production with no detectable change in the level of glycine resistance.

Introduction

Crown gall is a neoplastic disease of plants initiated by the specific activities of Agrobacterium tumefaciens (Smith and Townsend) Conn. The mechanisms by which the crown gall bacterium brings about the tumorous change in susceptible plant tissues are unknown. However, it is established that once the tumorigenic processes have occurred, the continued presence of detectable bacteria is not necessary for the proliferation of tumorous plant cells either in vivo or in vitro (3, 7, 28; see review, Ref. 6).

One approach to an understanding of the bacterial mechanisms involved in crown gall tumorigenesis is through the isolation and characterization of avirulent clones of the bacterium derived from initially pathogenic populations. Longley et al. (15) found that cultures of the crown gall organism could be routinely attenuated by growing them on media containing high concentrations of glycine. Van Lanen et al. (27) extended these findings to show that the loss of tumor-inducing ability also occurred during subculture on media containing the D isomers of several amino acids. Beardsley (1) reported that the attenuating amino acids select for resistant mutants.

Glycine, the D isomers of certain amino acids and several other agents interfere with mechanisms involved in bacterial cell wall synthesis (see reviews, Refs. 16, 26). In media which protect bacteria from osmotic disruption when they are exposed to such agents, viable spheric forms may be produced. Spheroplasts so formed from bacteria that are typically rod-shaped may revert to rods when transferred to media lacking the toxic agent, or, under certain circumstances, the conversion to spheroplasts may be irreversible (9).

Beardsley (2) suggested that the inability of attenuated, glycine-resistant clones of Agrobacterium tumefaciens to form tumors might reflect the failure of such bacteria to respond to spheroplast-inducing mechanisms within plant tissues. Rubio-Huertos and Desjardins (20) reported the production, within spheric bodies induced by exposure to glycine, of filterable forms of the crown-gall organism and suggested that these might be responsible for the secondary tumors that appear on some plants bearing a primary tumor. Subsequently, Rubio-Huertos and Beltra (19) reported the induction of primary crown-gall tumors by "fixed" spheric forms of Agrobacterium tumefaciens that had been obtained by culturing the organism on high concentrations of glycine.

The present paper reports experiments designed to examine the possibility that spheroplasts or filterable forms derived from spheroplasts, rather than the typical rod forms of the crown-gall bacterium, are the immediate agents of tumor induction. Spheroplasts and filterable forms may escape detection by ordinary bacteriologic methods (17, 21). Thus, evidence that tumorigenesis involves the conversion of rods into spheroplasts would reopen the question as to whether the tumorous growth in crown gall is dependent on the continuous presence of the bacterium in an as yet unidentified form or is independent of the bacterium.
Materials and Methods

Bacteria. *A. tumefaciens* strain B6 (23) was used throughout these experiments. Several glycine-resistant clones of the B6 strain were isolated by techniques previously reported (1). All stock cultures were maintained at 5°C on nutrient agar slants.

Media. 1. Liquid media: The glutamate medium described by Stonier (22) and modifications described below were used. In some experiments, nutrient broth (Difco) and the nutrient medium described by Lippincott and Heberlein (12) were also used.

2. Solid medium for plating: In poured plates, the bottom layer was solidified with 1.5% agar (Difco), the top layer with 0.7% agar. Bacteria, both rods and spheroplasts, were plated by suspending them in 2.5 ml of molten top-layer agar. This suspension was poured onto the surface of bottom-layered plates.

3. Chemicals and enzymes: Stock solutions of 1 M glycine (Eastman Organic Chemicals, Division of Eastman Kodak Co., Rochester, N. Y.) and d-methionine (Sigma Chemical Co., St. Louis, Mo.) were sterilized by membrane filtration (Millipore PH, 0.3 μ). Lysozyme and trypsin were kindly supplied by Dr. Moses Kunitz, Rockefeller University.

Pathogenicity determinations. 1. Routine host range studies: Five species of plants were used in the host range studies: *Kalanchoe daigremontiana* Ham et Perrier, tobacco (*Nicotiana tabacum* L., *N. rustica* L.), and Boston daisy (*Chrysanthemum frutescens* L.).

Plants were grown under greenhouse conditions, usually in clay pots. Routine inoculations were made by means of a single, transverse needle puncture into the middle of an internode about ¾ up the stem. Just prior to puncturing the stem, the sterile needle was scraped across the surface of an agar slant of a fresh culture of the bacteria to be tested. Tobacco plants were decapitated with a sharp razor by means of a single transverse cut across the upper quarter of an internode, about ¾ up the stem. Inoculations were then made about 1 cm below the decapsulation surface.

2. The relative tumor-forming efficiency of normal and spheroplast preparations: Spheroplasts were obtained by growing bacteria in the SP medium, described below, containing 0.02 M glycine. Control cultures were grown in the SP medium lacking glycine. Ten-ml samples of these cultures were centrifuged at approximately 4500 rpm for 30 min. Six ml of supernatant were removed, and the pellets were resuspended in the remaining 4 ml. Equal aliquots of supernatants and the concentrated, resuspended bacterial preparations were mixed in various combinations as shown in Table 1. Drops of the resulting suspensions were introduced into conditioned wounds (4) of *Kalanchoe*. Conditioning was achieved by puncturing stems with a sterile needle 48 hr prior to inoculation. The cicatrix was carefully removed prior to the introduction of bacteria. After inoculation, tumor induction was allowed to proceed at 25°C for various periods of time. At the end of the induction period, plants were placed at 32°C to inhibit further tumor induction (5, 11, 18). Tumors were scored independently by 2 of the authors by comparison with photographs of variously-sized tumors (11) 18 days after inoculation. Tumor scores covered the range from 0 (i.e., only wound
deciduous leaves of *Phaseolus vulgaris* L. var. pinto, developed by Lippincott and Heberlein (12, 13) for a quantitative determination of tumor-inducing ability were used.

Results

Formation of spheroplasts. Viable spheroplasts could not be produced in glutamate medium containing 0.02 M glycine even when the osmotic pressure of the medium was increased by adding sucrose at final concentrations between 0.1 and 20%. However, viable spheroplasts were induced by 0.02 M glycine in a modified medium prepared by omitting the citrate normally used to buffer glutamate medium. The modified medium supplemented with 0.5% sucrose is hereafter referred to as spheroplast (SP) medium. Eight hr after populations of bacteria growing in glutamate medium were inoculated into SP medium containing 0.02 M glycine, most of the cells, when observed by phase contrast microscopy, appeared to be normal, motile rods. A few club-shaped forms and an occasional motile, spheric form were also observed. After 24 hr, few normal-appearing cells remained. Clumps of nonmotile spheric forms, motile spheric forms, and motile deformed cells which were usually either club-shaped or rods markedly swollen in the center predominated. After 48 hr, nearly all of the cells were nonmotile spheroplasts, some of which were dividing. An occasional motile rod, presumably a glycine-resistant mutant, was found even after 72 hr.

Populations of spheroplasts, obtained after 72 hr of growth in glycine-supplemented medium, reverted to rods within 4 hr after transfer to glycine-free glutamate medium. Attempts to obtain permanent spheroplasts by continuous growth in SP medium containing 0.02 M glycine were unsuccessful. Growth of spheroplasts was maintained by transferring 1-ml samples from spher-
When spheroplasts were inoculated directly into wounds, shorter periods were required for tumor induction than when normal bacteria were used. For example, in one experiment, after 10 transfers, the ratio of spheroplasts capable of forming colonies on nutrient agar supplemented with 0.02 M glycine to colony-formers on unsupplemented medium was $2.1 \times 10^{-4}$. After 12 transfers, the ratio was $3.6 \times 10^{-4}$, and after 14 transfers, it was $8.1 \times 10^{-4}$. The latter ratio persisted through the 20th transfer.

Spheroplasts were also produced in SP medium supplemented with 0.005 M D-methionine. After 8 or 9 transfers in medium containing D-methionine, spheroplasts were obtained that did not revert to rods upon transfer to unsupplemented medium.

Both revertable and permanent spheroplasts were remarkably resistant to osmotic disruption. They could not be destroyed by abrupt transfer to either distilled water or distilled water containing sodium deoxycholate (0.5–5%). Further, no detectable effects on either the viability or morphology of revertable spheroplasts were observed following exposure to lysozyme (1 mg/ml) or trypsin (1 mg/ml).

The inhibition of spheroplast formation by citrate suggested that, as in the case of other bacteria (10), the processes involved require divalent ions. Viable spheroplasts could not be produced in nutrient broth supplemented with 0.02 M glycine. They were formed when this medium was also supplemented with $10^{-3}M$ MgSO$_4$. The similarity in rate of growth, as determined by changes in optical density, of 23-hr cultures of spheroplasts and rods in nutrient broth supplemented with $10^{-3}M$ MgSO$_4$ is indicated in Chart 1. The conversion of rods into spheroplasts occurred more rapidly in nutrient medium than in SP medium.

**Pathogenicity of Spheroplasts.** To test the possibility that spheroplasts might be highly efficient at inducing tumors, samples of centrifuged spheroplast cultures were inoculated into conditioned Kalanchoe wounds (4), in the presence and absence of normal bacteria. Bacterial suspensions were introduced into the wounds at intervals of about 60-90 min for a total of 6 applications, covering an 8-hr period. During this period, the plants were kept at 26.5°C. At the end of the 8-hr period, the plants were placed at 32°C to inhibit further tumor induction. Eight hr is a significantly shorter period than the minimum time required to produce tumors with normal bacteria (5). However, it was anticipated that if conversion of rods to spheroplasts is essential for tumor induction, the minimum period might be appreciably shorter when spheroplasts were inoculated directly into wounds. Neither the control suspensions, containing only typical rods, nor the spheroplasts induced tumors under the conditions employed.

To ascertain whether the spheroplast form of the organism was more or less efficient than the rod form at inducing tumors, 2 kinds of experiments were performed. In the 1st, populations of either spheroplasts or rods were inoculated into decapitated tobacco plants and permitted to act for 24, 44, and 48 hr under greenhouse conditions. In the 2nd, such preparations were introduced into conditioned Kalanchoe stem wounds and permitted to act for periods just long enough to produce small tumors.

In the experiments with decapitated tobacco plants, spheroplast preparations produced tumors at the inoculation punctures which were smaller than the tumors produced by suspensions of rods. In addition to the tumors at the inoculation site, tumors frequently develop at the nearby decapitation surface when, as in these experiments, the inoculation punctures are made just below the decapitation surface. In plants inoculated with the spheroplast preparations, tumors at the decapitation sites showed a tendency to form abnormal leaves and shoot as indicated in Fig. 1. These results suggest that spheroplasts are less efficient than typical rods at inducing tumors (24).

This suggestion was confirmed by results obtained in experiments in which spheroplasts and typical rods were permitted to act just long enough to initiate small tumors. In Kalanchoe, under the experimental conditions employed, maximum tumor induction occurred in conditioned wounds in about 25 hr at 25°C. Only occasional small tumors were formed under the same conditions in 20 hr. Spheroplast preparations mixed with supernatant from control cultures, when compared with normal bacteria mixed with glycine-containing supernatant from spheroplast cultures, proved to be less efficient at inducing tumors. The presence of glycine in the supernatant did not appreciably affect tumor induction. As indicated in Table 1, spheroplast cultures appeared to be considerably less efficient than bacteria with normal cell walls at inducing tumors in conditioned wounds of Kalanchoe stems.

None of the populations of permanent spheroplasts, obtained from cultures grown in medium supplemented with 0.005 M D-methionine, produced tumors in either Kalanchoe or Boston daisy.
TABLE 2

Effect of Glycine on Tumor-inducing Ability of A. tumefaciens, B6a

<table>
<thead>
<tr>
<th>Time after Addition of Glycine (hr)</th>
<th>Tumors/Leafb</th>
<th>0.02 M glycine</th>
<th>0.03 M glycine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Av.</td>
<td>N/N0</td>
<td>Av.</td>
</tr>
<tr>
<td>Control</td>
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<td>1</td>
<td>68.6</td>
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<td>0.25</td>
<td>50.3</td>
<td>0.93</td>
<td>48.3</td>
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<tr>
<td>0.5</td>
<td>51.7</td>
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<td>47.3</td>
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<td>0.73</td>
<td>25.5</td>
</tr>
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<tr>
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<td></td>
<td></td>
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</table>

a Glycine added to 48-hr culture grown in enriched nutrient broth (8, 12).
b No. of leaves = 16.

IMMEDIATE EFFECTS OF GLYCINE ON TUMOR-INDUCING ABILITY OF BACTERIAL POPULATIONS. Samples from 48-hr cultures of bacteria grown in nutrient broth supplemented with 0.1% yeast extract and 0.5% sucrose were diluted and plated onto nutrient agar and also inoculated into pinto-bean leaves at a 10^-8 dilution. Immediately after the samples had been removed, the cultures were supplemented with glycine at concentrations of either 0.02 or 0.03 M. At various times after glycine was added, samples were removed from the culture, diluted 1:10, and inoculated into primary pinto-bean leaves. Samples were also plated for viable cell counts. The decrease observed in the number of tumors formed after exposure of bacterial populations to glycine is indicated in Table 2. Over the time interval studied, no change in the number of viable bacteria was observed.

PATHOGENICITY OF GLYCINE-RESISTANT CLONES. Bacteria isolated from colonies growing on glycine-supplemented media frequently exhibit a loss of ability to induce tumors in the stem tissues of various hosts. Clones which had been plated only once on 0.02 M glycine-supplemented media usually showed no appreciable loss of virulence. Replating bacteria from such clones a 2nd time on media supplemented with 0.02 M or higher concentrations of glycine resulted in a dramatic loss of virulence either for all hosts tested, or for most hosts tested. A 3rd transfer to the same or higher concentrations of glycine usually resulted in complete avirulence. Loss of virulence during glycine attenuation, where tested, followed a pattern of host responses such as ability to induce tumors was lost first in Kalanchee, then in tomato and tobacco, and lastly in N. rustica and Boston daisy.

No correlation between rate of growth on glycine-supplemented media and virulence was observed. Some clones which grew well on glycine were substantially attenuated whereas others remained fully virulent. Further, some clones which grew poorly on glycine were almost completely avirulent, while others produced large tumors.

It was observed that during subculture and storage on media not supplemented with glycine, several clones which were initially completely attenuated for the hosts studied had regained their ability to produce tumors. In 1 instance, it was observed that the return to virulence occurred stepwise. In June 1961, clone MG-6, which previously had been completely attenuated, produced small tumors on N. rustica, but not on N. tabacum. By August of that year, the clone produced tumors on both N. rustica and N. tabacum.

To determine if the return of virulence was accompanied by a loss or decrease in glycine resistance, a clone that had recovered virulence was grown in glutamate medium and plated onto nutrient medium supplemented with 0.02 M and 0.03 M glycine. There was no detectable change in glycine resistance as determined by the ratio of colonies formed on glycine-supplemented plates to colonies formed on unsupplemented plates.

Discussion

The results presented here are not consistent with the hypothesis that crown gall tumorigenesis requires either spheroplasts of A. tumefaciens or filterable forms produced within spheroplasts. Permanent spheroplasts failed to produce tumors. Further, the tumor-inducing ability of populations of glycine-induced spheroplasts that retained the ability to revert to rods was consistently less than that of normal rods. Since tumors formed after infection with revertable spheroplasts may have been induced by bacteria that had reverted to rods, these findings suggest that the mechanisms by which the crown-gall bacterium produces tumors are not functional in spheroplasts.

This interpretation is consistent with the observation that the tumor-forming ability of bacterial populations exposed to glycine was reduced even before the morphologic changes associated with spheroplast formation were detectable. Within 30 min after the addition of glycine to cultures, the decrease in tumor-forming ability was evident and became more pronounced with continued incubation. This finding also indicates that tumor induction is not associated with a transient stage in the conversion of rods into spheroplasts. These considerations, combined with the observation that virulence may be regained within attenuated clones without a concomitant loss of glycine resistance, indicate that the failure of attenuated, glycine-resistant bacteria to form tumors cannot be explained by assuming that they are resistant to spheroplast-inducing agents similar to glycine within plant tissues.

Stonier et al. (25) recently demonstrated that growth and cell division are temporarily interrupted in populations of A. tumefaciens following heat shock and that the tumor-inducing ability of such populations is decreased for a corresponding period of time. Lippincott et al. (14) found that auxotrophic mutants of the crown-gall bacterium exhibit a lower capacity for producing tumors than the parental prototroph. They reported that the phenomenon of reduced virulence is a general characteristic of auxotrophic mutants rather than a characteristic limited to mutants requiring a specific substance or class of substances.

Thus, it appears that processes involved in normal bacterial growth and division are essential to tumor induction. Perhaps metabolism related to growth or bacterial components involved in cell-wall synthesis and division are altered in attenuated, glycine-resistant bacteria in such a manner than tumor-inducing mechanisms are either absent or nonfunctional. The return of virulence in some glycine-attenuated clones, without loss of glycine resistance, suggests that tumor-inducing mechanisms are not eliminated but are either blocked or masked in attenuated populations. A tumor-inducing mechanism clearly persists in population.

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Fig. 1. Abnormal shoot formed at decapitation site on Nicotiana tabacum by a spheroplast preparation inoculated into the internode below (arrow). Bacteria were permitted to act for 48 hr in the greenhouse before the plants were placed at 32°C. The tendency to organize was not observed when such tumors developed at the decapitation sites of internodes inoculated with control cultures.

...tions of some attenuated bacteria as evidenced by the observation that certain glycine-resistant clones which have lost the capacity for inducing tumors in some hosts retain the ability to produce tumors in other hosts.

Although not excluded by this study, the assumption that crown gall is an unusual form of bacterial hyperplasia in which an undetected form of the crown-gall organism enters plant cells and reproduces in synchrony with them lacks supporting evidence.

Acknowledgment

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References

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