*ON THE INHIBITION OF TUMOR INCEPTION IN THE CROWN-GALL DISEASE WITH THE USE OF RIBONUCLEASE A**

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The specific bacterium *Agrobacterium tumefaciens* (Smith and Town.) Conn can transform normal plant cells into tumor cells in short periods of time. Once the cellular transformation has been accomplished, the continued abnormal and autonomous proliferation of the affected host cells becomes an automatic process that is entirely independent of the inciting organism.[1-3] All of the many attempts that have been made in the past to isolate and characterize the tumor-inducing principle (TIP) that is elaborated by virulent bacteria and that is responsible for the persistent heritable change that occurs in the affected host cells have been unsuccessful. This may have resulted from the fact that (1) TIP is very labile and is rapidly inactivated either thermally or enzymatically, or (2) that more than one factor is involved in the transformation process and that those factors must be introduced into a host in proper sequence to be effective, or (3) the methods used for the isolation and the reintroduction of TIP into properly conditioned plant cells have not been sufficiently refined to accomplish the cellular transformation. Because of the repeated failures that have resulted from a direct approach to the problem of the isolation and characterization of TIP, indirect methods were applied in the present study in an attempt to gain insight into the nature of that principle. In these studies two enzymes, ribonuclease A and deoxyribonuclease, were used in an attempt to determine their effect on tumor inception and development. It is with a consideration of that aspect of the crown-gall tumor problem that the present study is concerned.

Experimental Methods.—It has been demonstrated that normal cells of the plant species *Kalanchoe daigremontiana* (Hamet et Perrier) may be converted into tumor cells by crown-gall bacteria at a temperature of 25°C but not at 32°C. Once the cellular transformation has been accomplished at the lower temperature, the tumor cells develop into neoplastic overgrowths equally well at both temperatures.[2, 5] The cellular transformation could thus be stopped abruptly and completely at any desired time following inoculation of plants with bacteria by placing and holding the inoculated plants at 32°C. The size and rate of growth of the tumors that developed at 32°C reflected the degree of cellular transformation that had taken place at the lower temperature prior to the time that the plants were placed and held at 32°C. It has been found, moreover, that the host cells must be conditioned as a result of irritation accompanying a wound if the cellular transformation is to occur. This conditioning process takes place independently of the bacteria at temperatures of both 25°C and 32°C, although the cellular transformation occurs only at the lower temperature.[4] Conditioning of the host cells takes place gradually, reaching a maximum susceptibility to transformation between the second and third days after a wound is made and then declines as wound healing progresses toward completion. These observations formed the basis of the experimental work reported here.

Kalanchoe daigremontiana (Hamet et Perrier) was used as the experimental test plant. The plants were grown in 4-in. pots and were used when approximately 10 cm tall. The experiments were carried out in two thermostatically controlled temperature chambers, one of which was maintained at 25 ± 0.5°C and the other at 32 ± 0.5°C. The relative humidity in both chambers was maintained at about 90%. Plants held at both 25°C and 32°C were kept under constant illumination by means of both fluorescent and incandescent lamps. Unless otherwise stated, the

results were recorded 14 days after the plants had been placed at 32°C for the final incubation at that temperature.

The B6 strain of *A. tumefaciens* was used in these studies. That strain was grown on Difco dextrose agar at 25°C for 24 hr. The bacterial inoculum was prepared just prior to use by making a heavy uniform suspension of a 24-hr culture of the bacteria in a modified yeast-extract-glucose-mineral-salts medium, as described by Pinckard.[6] In these studies Difco Bacto yeast extract was used in the amount of 3 gm per liter in place of the liquid yeast extract used by Pinckard.

Highly purified ribonuclease A and carboxymethyl histidine-119-ribonuclease were kindly supplied for these studies by Dr. Arthur M. Crestfield of the Rockefeller University. Electrophoretically pure deoxyribonuclease that was free of ribonuclease was purchased from the Worthington Biochemical Corp., Freehold, N. J. The deoxyribonuclease was dissolved in a 0.03 *M* $MgSO_4$ solution just before it was used.

Experimental Results.—In experiments designed to study the effects of deoxyribonuclease and ribonuclease A on tumor formation, *Kalanchoe daigremontiana* plants were wounded with a sterile dissecting needle into internodes that had fully elongated. Following wounding, the plants were placed in a thermostatically controlled chamber at 25°C for a period of 30 hr to allow the plant cells in the region of the wound to become conditioned and hence susceptible to transformation. In those studies enzyme solutions made up to the desired concentration were introduced directly into previously wounded areas with sterile dissecting needles between the 28th and 29th hr after the initial wound had been made. On the 30th hr following wounding, a heavy suspension of the bacteria was inoculated directly into the region of the wound. Thus, the enzyme solution was introduced into a plant 1–2 hr prior to the time that the plant was inoculated with the bacteria. Following inoculation the plants were allowed to incubate for 30 hr at 25°C, after which time they were placed and held at 32°C for a period of 14 days.

Deoxyribonuclease: Electrophoretically pure deoxyribonuclease used at concentrations up to 5 mg/ml was completely ineffective in inhibiting tumor inception or development when the enzyme solution was applied 1–2 hr prior to the time that the plants were inoculated with the bacteria or when the bacteria and enzyme solutions were added to the region of a wound simultaneously. The response obtained is illustrated in Figure 1*D*. A concentration as high as 5 mg/ml of deoxyribonuclease was used in these studies so that the number of units of specific activity of that enzyme would be comparable to that present in preparations containing the highest effective concentration of ribonuclease A used in these studies.

Ribonuclease A: In contrast to the results obtained with deoxyribonuclease, ribonuclease A used at a concentration of either 4 mg/ml or 2 mg/ml completely or almost completely inhibited tumor formation when the enzyme solution was applied 1–2 hr prior to the time that the wound site was inoculated with the bacteria. The occasional tumors that developed were small and they grew slowly when compared with those found on control plants, as shown in Figure 1*B*. Concentrations of the enzyme in the range of 1–0.5 mg/ml were also inhibitory, although the observed inhibition was not so pronounced as when higher concentrations of the enzyme were used. When 0.1 mg/ml or 0.05 mg/ml of ribonuclease A were applied, there did not appear to be any significant inhibition of tumor inception or development. The results obtained in one representative experiment are shown in Figure 2.

Since the concentration of ribonuclease A required to inhibit tumor formation was unusually large, the possibility existed that it was not the catalytic effects of the enzyme that were responsible for the suppression of tumors but rather that the

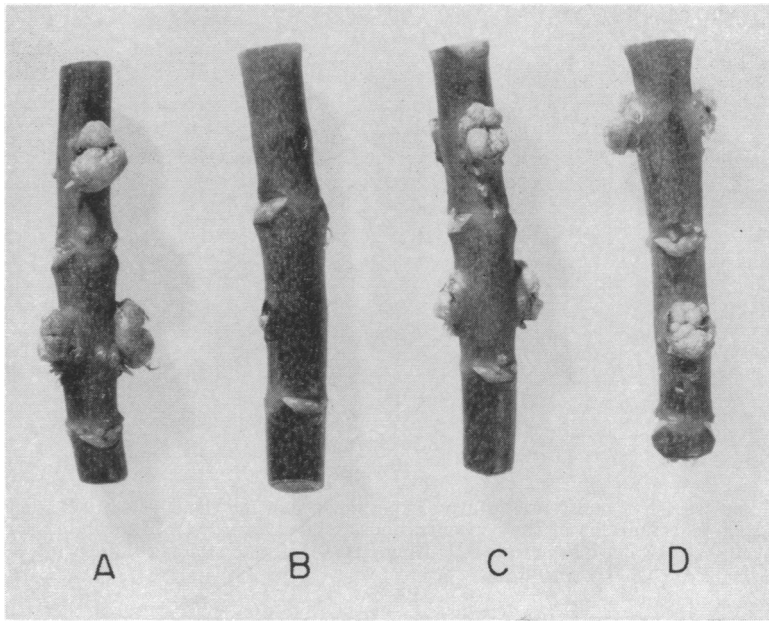

FIG. 1.–Effect of ribonuclease A, carboxymethyl histidine-119-ribonuclease, and deoxyribonuclease on tumor inception and development. (*A*) Inoculated control; (*B*) ribonuclease A; (*C*) carboxymethyl histidine-119-ribonuclease; (*D*) deoxyribonuclease.

presence of relatively high concentrations of a basic protein was somehow involved. In order to test that possibility, the carboxymethyl histidine derivative of ribonuclease A was used. That compound does not possess ribonuclease activity and differs from ribonuclease A only in that the histidine in the 119 position of the molecule had been blocked by alkylation. When carboxymethyl histidine-119-ribonuclease was used at a concentration of 4 mg/ml and applied to conditioned cells 1–2 hr prior to inoculation, it did not inhibit tumor inception or development, and the resulting tumors (Fig. 1*C*) were comparable to those found in the control plants (Fig. 1*A*).

The results reported above suggest that ribonuclease A affects (1) the bacteria, (2) the host cells, or (3) exerts its inhibitory effects in some other way, possibly by inactivating the tumor-inducing principle itself.

Effect of ribonuclease A on the bacteria: In order to learn whether ribonuclease A exerted an inhibitory effect on bacterial replication, studies were carried out both in culture and in the plant. In the cultural studies the bacteria were grown for 24 hr on Difco dextrose agar slants. A dilute suspension of the bacteria was made up in yeast-extract-dextrose-mineral-salts medium and one drop of that suspension was placed in flasks containing that medium or that medium having 4 mg/ml of ribonuclease A. In both instances the media were sterilized prior to inoculation with bacteria by filtration through Millipore (HA 0.45 μ pore size) filters. Following inoculation, the flasks were placed on a reciprocal shaker and turbidity was measured at 540 mμ periodically during a 96-hr period with the use of a Beckman DU spectrophotometer. The results of those studies, which are shown in Figure 3, demonstrate that throughout the duration of the experiment no significant dif-

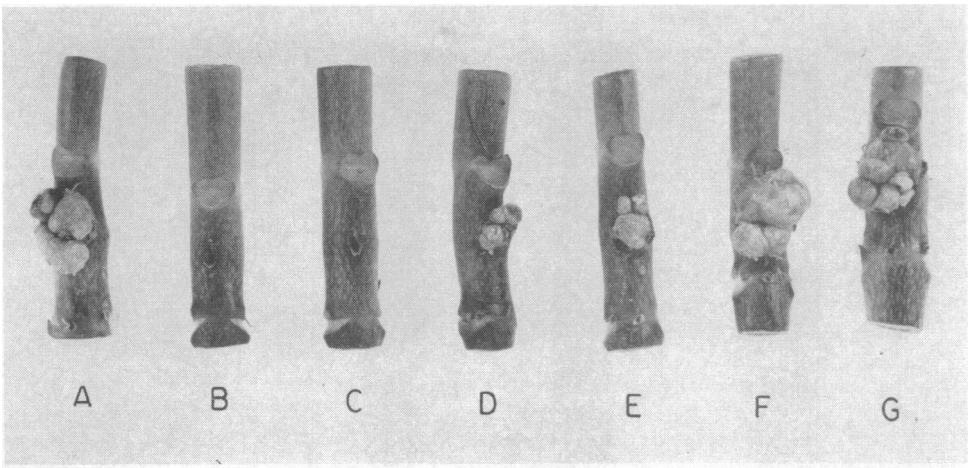

FIG. 2.—The results of one representative experiment showing the effects of ribonuclease A on tumor inception as a function of the concentration of the enzyme. (*A*) Inoculated control; (*B*) 4 mg RNase/ml; (*C*) 2 mg RNase/ml; (*D*) 1 mg RNase/ml; (*E*) 0.5 mg RNase/ml; (*F*) 0.1 mg RNase/ml; (*G*) 0.05 mg RNase/ml.

ference in the rate or amount of growth could be detected between the control medium and that medium containing ribonuclease A.

In order to learn whether ribonuclease A exerted an inhibitory effect on bacterial replication in a host plant, one drop of bacterial suspension, containing about 20,000 viable cells per drop, and made up in yeast-extract-dextrose-mineral-salts medium containing 4 mg/ml ribonuclease A, was introduced with a dissecting needle into internodes of *Kalanchoe* plants. As a control, similar numbers of bacteria were suspended in the same medium without enzyme and introduced into the host plants in the same way. The plants were incubated in a chamber at 25°C. Bacteria were isolated from wounded areas in both instances after 1, 24, 48, 72, and 96 hr. In order to study the replication of the bacteria in wounded areas treated with ribonuclease A and in control plants, tissues surrounding a wound were isolated, thoroughly triturated in a sterile mortar containing 1 ml of yeast-extract-dextrose-mineral-salts broth, and plated in Petri dishes in Difco dextrose agar. The plates were incubated at 25°C and bacterial counts were made 7 days after plating. The results of those studies showed that the bacteria multiplied as well throughout the 4-day period in the presence of ribonuclease A as they did in the control plants. Thus, the presence of that enzyme at the concentration used did not affect the replication of the bacteria in the host.

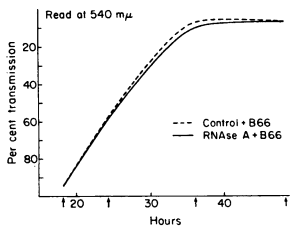

FIG. 3.—Growth curves showing that no significant difference is found when bacteria are grown in a medium containing ribonuclease A at a concentration of 4 mg/ml or in that medium without added enzyme.

In order to test the effect of ribonuclease A on the ability of the bacteria to initiate tumors, the bacteria were grown in filter-sterilized yeast-extract-dextrose-mineral-salts medium containing 4 mg/ml of ribonuclease A for a period of 24 hr. In controls the bacteria were grown in that medium without enzyme for the same period of time. Following incubation at 25°C the

bacteria in both instances were centrifuged, the supernatant was decanted, and the bacterial pellet resuspended in a volume of the basic (enzyme-free) medium. This procedure was repeated a second time. The bacteria in each instance were then inoculated directly into wounds that had been allowed to heal for 30 hr. Following a 30-hr incubation period at 25°C, the plants were placed and held at 32°C for 14 days, after which time the results were recorded. The results of those studies showed that exposure of the bacteria to ribonuclease A for 24 hr had no significant effect on the ability of the organism to initiate tumors when compared with bacteria grown on the same medium in the absence of that enzyme.

Since ribonuclease A did not appear to affect the growth of the bacteria *in vitro* or in a host plant, nor did it appear to affect the virulence of the bacteria, an attempt was made to learn whether that enzyme interfered with the normal wound-healing response in the host. Since differences in the rate or extent of the wound-healing response in enzyme-treated as compared with control tissues could well account for the inhibitory effects observed on tumor formation, a histological study was undertaken to determine whether any differences in the wound response could in fact be demonstrated histologically. In those studies ribonuclease A (4 mg/ml) was introduced with a dissecting needle into internodes of *Kalanchoe* stems at the time of wounding. In other studies, sterile wounds were made with a needle and the wounds were allowed to heal for 30 hr at 25°C, after which time ribonuclease A (4 mg/ml) was introduced directly into the previously wounded area. As controls in this experiment, a drop of water was used in place of the enzyme solution in the two instances described above.

Tissue from the region of the wound in the four treatments described above was fixed in formalin-acetic acid-alcohol 2, 3, and 4 days after the initial wound was made. Following fixation, the tissues were dehydrated, cleared, embedded in paraffin, sectioned, and stained with safranin. The results of those histological studies showed that there was no significant difference in either the rate or the extent of the wound-healing process between tissues that had been treated with the enzyme and those treated with water. The extent of the wound-healing response was measured by the number of new cell divisions that had occurred in the cortex, vascular region, and pith. Thus, the presence of the enzyme did not appear to affect the capacity of the host cells to respond in a normal manner to the stimulus of a wound.

In order to learn whether any stage in the normal wound-healing cycle is particularly vulnerable to the action of ribonuclease A, *Kalanchoe* plants were inoculated with one drop of a heavy suspension of the crown-gall bacteria and placed in a chamber at 25°C. At intervals of 6, 12, 18, 24, 36, 48, and 72 hr following inoculation, two plants each were treated with ribonuclease A at a concentration of 2 mg/ml. Control plants inoculated with bacteria received distilled water in place of the enzyme solution at the time intervals indicated above. The results of those studies showed that no significant differences in the size and rate of growth of tumors initiated in the presence of the enzyme and those that developed in the control plants were found. It thus appears that the enzyme does not interfere significantly with the transformation process at any stage in the normal wound-healing cycle provided the bacteria are allowed to occupy certain of the intracellular spaces of the host prior to the time that the enzyme solution is added to the tissues. The results of this study emphasize, furthermore, the need for having the enzyme solution

present in the tissues prior to the time that the bacteria are applied if maximum inhibition of tumor inception is to be achieved. It would appear that the enzyme solution must thoroughly coat the cell walls surrounding the intracellular spaces in the region of a wound before the bacteria are added if the enzyme is to be effective.

Discussion and Conclusions.—The results reported in this study indicate that ribonuclease A, but not deoxyribonuclease, inhibited significantly the inception of tumors in the crown-gall disease of plants. Ribonuclease A did not affect the growth rate of the inciting bacteria either in culture or in a host. No differences could be observed, moreover, in the rate or extent of the wound-healing response when cells treated with ribonuclease A were compared with untreated cells in the region of a wound. These results suggest either that the tumor-inducing principle itself or an essential component of that principle is a ribonucleic acid, or that ribonuclease A enters the bacteria or the host cells and in either case selectively inactivates some component essential for tumor inception without, however, affecting the capacity of such cells to grow and divide at a rate comparable to that of untreated cells.

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