Ribonuclease-Induced Alterations in Agrobacterium tumefaciens

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Crown gall tumors are initiated during contact between the wounded tissues of susceptible plants and cells of a virulent strain of *Agrobacterium tumefaciens*. The tumorigenic property of the bacterium is hypothesized to exist in a tumor-inducing principle (TIP) (1). The nature of this hypothetical TIP and the mechanism of the tumorigenic process remain unknown.

Braun & Wood (3) reported that ribonuclease (RNase) when added to the wound site prior to bacterial inoculation resulted in marked inhibition of tumor inception. The enzyme was without apparent effect on rate and extent of bacterial growth or on wound response in the host. These experiments suggested that the inhibitory action of RNase was due to selective inactivation of some bacterial or host component essential for tumor inception.

By further exploration of the action of RNase on *A. tumefaciens*, we demonstrated the occurrence of pronounced alterations of some properties of the bacterial cells. These findings, while not necessarily precluding the possibility that TIP involves ribonucleic acid, may provide an alternate hypothesis for explaining the inhibitory property of RNase on tumor inception.

Ribonuclease A (phosphate-free) with a designated activity of 2,500 units/mg, was purchased from Nutritional Biochemicals Corp. Aqueous solutions of the enzymes were heated for 10 min at 70 C to inactivate any heat-labile enzyme impurities which may have been present.

We harvested cells of *A. tumefaciens* during the exponential growth phase and transferred them into a chemically defined medium (4) supplemented with leucine-^{14}C, uracil-^{14}C, or thymidine-^{14}C at a level of 0.05 μc/ml (specific activity: 2.5 μc/μM). After a prelabeling period of sufficient duration to establish linearity of precursor uptake, the cultures were divided. To one half was added a heated RNase solution to give a final concentration of 4 mg/ml, and to the remainder was added an equal amount of distilled water. Incorporation of the labeled compound was followed over a 40-min period, and was expressed as counts/min in the fraction insoluble in trichloroacetic acid. Counting was done with a thin-window GM counter.

The rates of incorporation of the labeled compounds into the nontreated cells remained linear during the test period. The enzyme-treated cells, in contrast, showed marked increases in incorporation rates (Fig. 1). The

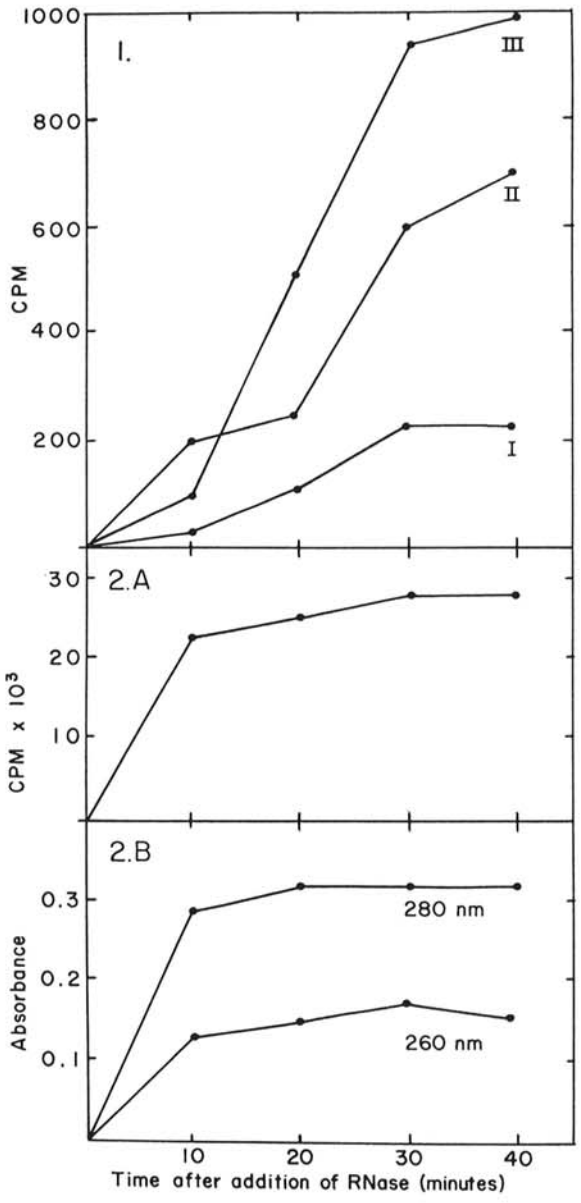

Fig. 1-2. 1) Effect of ribonuclease (RNase) on rates of incorporation of thymidine-^{14}C (I), leucine-^{14}C (II), and uracil-^{14}C (III) by cells of *Agrobacterium tumefaciens*. Each point represents the counts/min (CPM) in the trichloroacetic acid insoluble fraction of RNase-treated cells. All points have been corrected for the CPM of nontreated cells. **2) A)** Kinetics of release of radioactivity from cells of *Agrobacterium tumefaciens* treated with RNase. Cells were prelabeled for 48 hr with uracil-^{14}C. Each point represents the counts/min (CPM) in the filtrate from enzyme-treated cells. All points have been corrected for the CPM of control cells. **B)** Kinetics of release of ultraviolet-absorbing materials from cells of *Agrobacterium tumefaciens* treated with RNase. Each point represents the absorbancy of the filtrate from an RNase-treated cell suspension corrected both for control readings and for the absorbance due to enzyme protein.

rate of leucine-^{14}C incorporation increased threefold, that of thymidine-^{14}C increased fivefold, and uracil-^{14}C was incorporated at a rate 12 times that of its control.

This apparent enhancement of the rate of macromolecular synthesis suggested that RNase altered the metabolism of *A. tumefaciens*. We next determined whether RNase also was able to induce leakage of macromolecular degradation products from the bacterial cells. *A. tumefaciens* was grown for 48 hr on slants of chemically defined medium (4) containing 10 μc of leucine-^{14}C, uracil-^{14}C, or thymidine-^{14}C. The cells were harvested, incubated for half a generation time (60 min) in media supplemented with the respective unlabeled compound, and washed twice with cold, sterile physiological saline. The cells were then suspended in an RNase solution as described above. At various time intervals, aliquots of the cell suspension were filtered and washed on membrane filters. The radioactivity in the filtrate was measured with a GM counter. Figure 2-A illustrates typical results obtained using cells prelabeled with uracil-^{14}C. Within 10 min following enzyme treatment, the amount of released radioactivity constituted over 20% of the incorporated label. Similar results were obtained from RNase-treated cells prelabeled with thymidine-^{14}C. The enzymatic release of radioactivity from cells prelabeled with leucine-^{14}C, however, was rapidly obscured by spontaneous leakage of labeled compounds, probably metabolic products of leucine.

We next measured enzymatic release of cellular substances which absorb in the ultraviolet range. Filtrates from cell suspensions treated with RNase contained appreciable amounts of materials absorbing at 260 nm and 280 nm (Fig. 2-B). The presence of these substances in the filtrates implicated enzyme-induced leakage of intracellular components, presumably protein or nucleic acids or their degradation products. The amount of such materials released by RNase was equivalent to 70% of the compounds soluble in perchloric acid, thus reflecting either a considerable depletion of intracellular precursors or an active excretion of breakdown products. The kinetics of release of these ultraviolet-absorbing materials closely paralleled the release of radioactivity from cells prelabeled with uracil-^{14}C or thymidine-^{14}C.

Cells of *A. tumefaciens* undergo certain alterations during exposure to RNase. These alterations are manifested by (i) increased rates of incorporation of macromolecular precursors; (ii) release of radioactivity from prelabeled cells; and (iii) leakage of ultraviolet-absorbing components. These effects reflect changes in the bacterial cells that are too subtle to measure in terms of altered growth rates or decrease in viable cell count. Braun & Wood (3) could not detect an inhibitory effect of RNase on the growth of *A. tumefaciens* either in media or in the host tissue. We also were unable to detect any diminution in viable counts during exposure of *A. tumefaciens* to RNase for 24 hr.

It is believed that formation of a crown gall tumor requires a defined sequence of interactions between cells of *A. tumefaciens* and a receptive host tissue (2). Any disruption of these sequential events may interfere with the tumorigenic process. The alterations we observed in cells of *A. tumefaciens* during exposure to RNase may be sufficient to disrupt the synchronous host-pathogen interactions requisite for tumor inception. These experiments support the hypothesis that RNase acts as a nonspecific inhibitor of crown gall tumorigenesis by modifying macromolecular synthesis and degradation in *A. tumefaciens*. The inhibitory action of RNase on tumor inception, if nonspecific, does not provide a basis for deducing the chemical nature of the tumor-inducing principle of *A. tumefaciens*.

LITERATURE CITED

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