Effects of 5-Fluorouracil and 5-Fluorodeoxyuridine on Growth and Tumor-Inducing Ability of *Agrobacterium tumefaciens*

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ABSTRACT

BEARDSLEY, ROBERT E. (Manhattan College, Bronx, N.Y.), and JACQUES LIPETZ Effects of 5-fluorouracil and 5-fluorodeoxyuridine on growth and tumor-inducing ability of *Agrobacterium tumefaciens*. J. Bacteriol. 92:346-348. 1966.—*Agrobacterium tumefaciens* B6, grown in the presence of 5-fluorouracil or 5-fluorodeoxyuridine, exhibited a prolonged lag phase. The tumor-inducing ability of bacteria grown in the presence of these compounds was decreased even after exposures as short as 40 min. A positive correlation was found between the growth-inhibitory effects of these compounds and their effects on the tumor-inducing ability of the bacteria.

The mechanisms by which *Agrobacterium tumefaciens* (Smith and Townsend) Conn induces the tumorous changes characteristic of crown gall in susceptible plant tissues are unknown. Several lines of evidence suggest that these mechanisms include the elaboration by the bacterium of a specific tumorigenic agent which Braun designated as the "tumor-inducing principle" (TIP). However, attempts to isolate, to identify, or even to demonstrate unequivocally the existence of TIP have so far been unsuccessful [see review by Braun (3)].

It is of considerable interest therefore that halogenated pyrimidines and ribosides have been reported to inhibit specifically crown gall tumorigenesis. Bopp (1) reported that when either 5-fluorouracil (FU) or 5-fluorodeoxyuridine (FDU) was introduced into infected plant tissues, at concentrations of 200 and 10 $\mu\text{g}/\text{ml}$, respectively, tumor formation was inhibited. Subsequently, he concluded that a specific deoxyribonucleic acid (DNA) participates in the formation of tumors (2). Although Bopp (1, 2) reported that neither FU nor FDU altered the pathogenicity of the bacteria, Lang and Baker (4) reported that another halogenated pyrimidine, 5-iododeoxyuridine caused a loss of virulence of *A. tumefaciens*.

The present paper reports experiments designed to explore the possibility that FU and FDU might provide unequivocal evidence regarding the nature of TIP. Since the interpretation of in vivo studies is complicated by possible effects on

host cells, bacteria were treated with these compounds in vitro.

MATERIALS AND METHODS

A. tumefaciens strain B6, originally obtained from Armin C. Braun of The Rockefeller University, was used. Bacteria were grown in glutamate medium (9). Viable-cell counts were made by plating onto nutrient agar (Difco). Growth studies were carried out by measuring changes in optical density, at 600 $m\mu$, of freshly inoculated cultures. Aqueous solutions of FU and FDU, 0.1 mg/ml (Hoffman La Roche, Inc., Nutley, N.J.), were sterilized by membrane filtration (50- $m\mu$ pore size; Millipore Filter Corp., Bedford, Mass.) before being added to cooled, autoclaved medium.

Tumor-inducing ability was measured by a modification, described below, of the quantitative pinto bean assay developed by Lippincott and Heberlein (6).

RESULTS

Effects of FU and FDU on the growth of bacteria. The effects of FU and FDU on the growth of bacteria are shown in Fig. 1. Although the duration of the lag in growth produced by these compounds varied in different experiments, the initiation of growth was inhibited by concentrations of 0.2 $\mu\text{g}/\text{ml}$. The growth rate was depressed by concentration's of 2.0 $\mu\text{g}/\text{ml}$.

To evaluate the variability among bacteria in the ability to grow in medium containing the halogenated compounds, populations growing in glutamate medium were plated onto solidified medium (1.5% agar) supplemented with various

concentrations of either FU or FDU. The resulting colonies were counted, and numbers were compared with the number of colonies on unsupplemented medium. The results (Table 1) indicate that, in the presence of 0.125 $\mu\text{g}/\text{ml}$ and higher concentrations of FU, only about 0.01% of the

FIG. 1. Growth of *Agrobacterium tumefaciens* in medium supplemented with 5-fluorouracil (A) and 5-fluorodeoxyuridine (B). Symbols: ○, control; ●, 0.2 $\mu\text{g}/\text{ml}$; △, 2.0 $\mu\text{g}/\text{ml}$.

TABLE 1. Effect of 5-fluorouracil (FU) and 5-fluorodeoxyuridine (FDU) on survival of *Agrobacterium tumefaciens* B6

FU	N/N ₀ ^a	FDU	N/N ₀
$\mu\text{g}/\text{ml}$		$\mu\text{g}/\text{ml}$	
0.125	2.9×10^{-4}	0.125	1
0.25	1.6×10^{-4}	0.25	1
0.5	1.2×10^{-4}	0.5	1
1.0	9.5×10^{-5}	1.0	0.71
2.0	1.0×10^{-4}	2.0	0.58

^a No. of colonies on supplemented medium/no. of colonies on control plates.

plated population gives rise to detectable colonies. The fraction of plated bacteria selected as colony formers by FDU is significantly larger. Over 50% of the population grows to produce colonies on medium supplemented with 2.0 $\mu\text{g}/\text{ml}$ of this compound.

Preliminary experiments were conducted to determine whether bacteria surviving as colony formers on plates supplemented with FU were more resistant than the original population to the toxic effects of this compound. Bacteria were isolated from colonies growing on plates containing 2.0 $\mu\text{g}/\text{ml}$ of FU and streaked onto nutrient agar slants. Cultures in glutamate medium were inoculated with bacteria from slants. Samples from these cultures were plated onto medium supplemented with FU. The clones tested were resistant, as indicated by the finding that over 50% of the bacteria in plated samples grew to form colonies on medium containing 2.0 $\mu\text{g}/\text{ml}$ of FU.

Effects of FU and FDU on tumor-inducing ability. An 18-hr culture growing in glutamate medium was divided into four equal portions of 25 ml each. One served as a control. The remaining three were supplemented with FU and FDU to give the final concentrations indicated in Table 2. At 40 min and at 26 hr after addition of the compounds, 5-ml samples were removed from each culture. The bacteria in these samples were sedimented by centrifugation and were resuspended in fresh, unsupplemented medium. Samples from the resuspended populations were diluted and plated. Additional 0.1-ml samples were inoculated into primary pinto bean leaves. As indicated in Table 2, the tumor-inducing ability (TIA) of bacterial populations decreased within 40 min after addition of either FU or FDU. After 26 hr of exposure to FU, the TIA

TABLE 2. Effect of 5-fluorouracil (FU) and 5-fluorodeoxyuridine (FDU) on tumor-forming ability of *Agrobacterium tumefaciens* B6

Treatment period	Tumor formation					
	FU, 0.2 $\mu\text{g}/\text{ml}$		FU, 2.0 $\mu\text{g}/\text{ml}$		FDU, 2.0 $\mu\text{g}/\text{ml}$	
	TFU ^a	Per cent SI ^b	TFU ^a	Per cent SI ^b	TFU ^a	Per cent SI ^b
40 min ^c	6.67×10^7	50	4.58×10^7	73	1.74×10^8	19
26 hr ^d	1.14×10^9	3.4	2.0×10^9	2.0	5.76×10^7	68

^a TFU (tumor-forming unit) = $\frac{\text{no. of bacteria inoculated/leaf} (\times 10^4)}{\text{average no. of tumors/leaf}}$

Number of leaves scored per TFU determination = 20.

^b Per cent SI (% specific infectivity) = $\frac{\text{TFU of control}}{\text{TFU of treated culture}} \times 100$.

^c TFU (control) = 3.33×10^7 .

^d TFU (control) = 3.90×10^7 .

was further reduced to a small fraction of that of the untreated control. However, after 26 hr, the TIA of bacteria exposed to FDU more closely approximated that of the control than it did after 40 min.

Viable-cell counts indicated that, during the 26-hr period, the initial population of 1.8×10^9 bacteria per milliliter increased to 3.2×10^9 with 0.2 $\mu\text{g/ml}$ of FU), to 3.5×10^9 with 2.0 $\mu\text{g/ml}$ of FU), and to 5.6×10^9 cells per milliliter with 2.0 $\mu\text{g/ml}$ of FDU. The control did not increase.

DISCUSSION

Evidence for a specific effect of FU and FDU on the tumor-inducing mechanisms of *A. tumefaciens* was not obtained in this study. Although these compounds markedly decreased the tumor-inducing ability of the bacterium, the concentrations employed were inhibitory to bacterial growth. It was not possible to dissociate effects on tumor-inducing ability from effects on growth. In fact, there was a positive correlation between these two phenomena.

Thus, the results are consistent with the hypothesis, suggested by several lines of evidence (5, 7, 8, 10), that metabolism related to bacterial growth is essential for tumor induction. The results provide no information by which to interpret the findings reported by Bopp (1, 2) for his *in vivo* studies, but reaffirm the necessity for excluding effects on bacterial growth before attributing inhibition of tumor induction to specific mechanisms.

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LITERATURE CITED

1. BOPP, M. 1961. Die Bedeutung der DNA für die primäre Zellveränderung bei Induktion von Wurzelhalsgallen. *Z. Naturforsch.* **16b**:336-347.
2. BOPP, M. 1964. Induction tumorale et son inhibition. *Bull. Soc. Franc. Physiol. Vegetale.* **10**:18-31.
3. BRAUN, A. C. 1962. Tumor inception and development in the crown gall disease. *Ann. Rev. Plant Physiol.* **13**:533-558.
4. LANG, J., AND R. BAKER. 1966. Influence of compounds with antitumor activity on crown gall. *Phytopathology* **56**:131-133.
5. LIPETZ, J., AND T. STONIER. 1961. Effect of 5-bromuracil on crown gall tumour induction. *Nature* **190**:929-930.
6. LIPPINCOTT, J. A., AND G. T. HEBERLEIN. 1965. The quantitative determination of the infectivity of *Agrobacterium tumefaciens*. *Am. J. Botany* **52**:856-863.
7. LIPPINCOTT, J. A., AND B. B. LIPPINCOTT. 1965. Timing of events in crown-gall tumor development on pinto bean leaves. *Develop. Biol.* **12**:309-327.
8. LIPPINCOTT, J. A., J. H. WEBB, AND B. B. LIPPINCOTT. 1965. Auxotrophic mutation and infectivity of *Agrobacterium tumefaciens*. *J. Bacteriol.* **90**:1155-1156.
9. STONIER, T. 1956. Labeling crown gall bacteria with P^{32} for radioautography. *J. Bacteriol.* **72**:259-268.
10. STONIER, T., R. BEARDSLEY, L. PARSONS, AND J. MCSHARRY. 1966. *Agrobacterium tumefaciens* Conn. III. Effect of thermal shock on bacteria in relation to tumor-inducing ability. *J. Bacteriol.* **91**:266-269.