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In Vitro Binding of *Agrobacterium tumefaciens* to Plant Cells from Suspension Culture[1]

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ABSTRACT

In vitro **binding experiments were carried out using ^{32}P-labeled cells of the virulent *Agrobacterium tumefaciens* strain B6 and *Datura innoxia* cells from suspension culture. Binding kinetics showed that adherence of bacteria to *Datura* cells increased gradually during the first 60 minutes and attained a maximum level within 120 minutes of incubation. Maximum binding occurred at pH 6.0. The presence of Ca^{2+} and Mg^{2+} reduced binding slightly and EDTA had little effect at concentrations of 0.1 to 10 millimolar. The binding of bacteria to *Datura* cells was temperature-dependent. *Escherichia coli, Salmonella typhimurium, Rhizobium japonicum,* and *Micrococcus lysodeikticus* did not compete with virulent *A. tumefaciens* strain B6 for binding to *Datura* cells. The admixture of avirulent *A. tumefaciens* strain IIBNV6 enhanced adherence of virulent *A. tumefaciens* strain B6 to *Datura* cells. Octopine had no effect on the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells, but 10 millimolar canavanine was inhibitory. Arginine enhanced the adherence of the bacteria at concentrations higher than 0.1 millimolar. Incubation with DNase, RNase, and lipase did not affect the binding, but protease stimulated the adherence of bacteria to *Datura* cells. Concanavaline A and soybean lectin had little effect whereas lecithin and lysolecithin enhanced binding slightly. Poly-L-lysine markedly stimulated the bacteria-plant cell adherence. Cells from suspension cultures of pea, vetch, and soybean had a 2- to 3-fold higher binding capacity than *Datura* cells, whereas cells from wheat, corn, rice, and sorghum had a considerably lower affinity for binding with virulent *A. tumefaciens* strain B6. Bacterial adherence to plant cells was confirmed by autoradiography and electron microscopy. Autoradiographic analysis showed that bacteria were associated with the cell wall, and that often binding of bacteria was localized. Electron micrographs clearly illustrated a tight association of virulent *A. tumefaciens* strain B6 cells to the *Datura* cell wall.**

Crown gall tumor induction by *Agrobacterium tumefaciens* requires a specific association between the bacteria and plant wound site (18). Subsequently, the tumor-inducing principle (TIP) (7) can be transferred from the bacteria to the host plant cell (33, 37). Studies on the bacterial interaction with plant cells have been carried out by *in vivo* inoculating of the bacteria on primary leaves (15), seedlings (34), stems (3), and root discs (36). However, tissue culture may provide more defined, reproducible and quantitative systems which is necessary for biochemical studies on bacteria-plant cell association.

The purpose of this paper is to provide quantitative data and biochemical information on the attachment of virulent *A. tumefaciens* strain B6 to plant cells from suspension cultures. Availability of cell suspension cultures made it possible to investigate the quantitative kinetics of the adherence of bacteria to plant cells.

MATERIALS AND METHODS

Plant Cells. *Datura innoxia* cells were cultured in 1-M51C medium containing (per liter): KNO_3, 2 g; NH_4NO_3, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.37 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; KH_2PO_4, 275 mg; EDTA-Fe, 40 mg; N-Z amine (type A, Hunko-Sheffield), 0.2 g; L-glutamine, 292 mg; sucrose, 30 g; vitamins, micronutrients, KI, and 2,4-D as in B5 medium (28). Two-day-old cells were washed and suspended in 1-B5 medium.

Bacterial Strains. *A. tumefaciens* strains (virulent and avirulent) were obtained from A. C. Braun, The Rockefeller University, New York. The cultures were grown in liquid medium containing Nutrient Broth (8 g/l, Difco) or agar medium containing Dextrose Agar (43 g/l, Difco).

Labeling of Virulent *A. tumefaciens* Strain B6 with [^{32}P]Orthophosphate or with [^{14}C]Adenine. Bacteria were grown in Nutrient Broth containing [^{32}P]orthophosphate (2 μCi/ml, New England Nuclear) at 28 C overnight. The cells were washed with 10 mM K-phosphate (pH 6.0) and suspended in the same buffer (approximately 3.0×10^9 cells/ml). Specific radioactivity was 2×10^{-3} cpm/cell to 8×10^{-4} cpm/cell. For the study of autoradiography, *A. tumefaciens* strain B6 was grown in Nutrient Broth containing [^{14}C]adenine (3 μCi/ml, New England Nuclear) at 28 C overnight. The cells were washed with 10 mM K-phosphate (pH 6.0) and suspended in the same buffer (2.3×10^9 cells/ml, 0.9×10^{-3} dpm/cell).

Standard Binding Mixture and Determination of Binding. The standard binding mixture was 1.5 ml, consisting of 1.0 ml *Datura* cell suspension (1–4 mg dry wt/ml), 0.1 ml ^{32}P-labeled virulent *A. tumefaciens* strain B6 (3.0×10^8 cells), 0.15 ml 0.1 M K-phosphate (pH 6.0), and 0.25 ml distilled H_2O. The mixture was incubated for 2 hr at 28 C on a shaker. The plant cells in the binding mixture were transferred to a Miracloth disc (2.1 cm diameter) attached to a Millipore filter apparatus and washed with 5-ml portions of 1-B5 medium three times. The Miracloth disc with cells was then transferred into a scintillation vial and counted with 5 ml of toluene-Triton X-100 (2:1) containing 5 g/l butyl-PBD in a Searls Mark III liquid scintillation counter.

Autoradiography of Virulent *A. tumefaciens* Strain B6 Bound to *Datura* cells. After incubation with [^{14}C]adenine-labeled *A. tumefaciens* strain B6, *Datura* cells were washed five times with 1-B5 medium. The cells were then fixed in 10% (v/v) formaldehyde in 10 mM Na-phosphate (pH 7.4) for 5 days at 4 C. A drop of cell suspension was placed on a glass slide and a coverslip was firmly

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pressed on top of the cells. After removal of excess liquid the slide with coverslip facing down was placed on solid dry ice. The coverslip was removed and the slide allowed to air dry for several hr. The slide was then dipped in Kodak NTB-3 emulsion at 37 C and allowed to air dry for 2 hr. After exposure for 72 hr the preparation was developed, fixed, and the cells stained with a solution of 0.01% fast green in 2% acetic acid and examined microscopically.

Electron Microscopy of Virulent *A. tumefaciens* Strain B6 Bound to *Datura* Cells. The techniques for electron microscopy were modified from those described in detail elsewhere (11). Cells were fixed at room temperature in 1% glutaraldehyde for 1 hr followed by 3% glutaraldehyde for 2 hr. The glutaraldehyde was prepared in 25 mM Na-phosphate (pH 6.8). The cells were washed with buffer (five changes over 2.5 hr) and then postfixed in 1% osmium tetroxide in the phosphate buffer overnight at 0 C. After a brief wash the cells were slowly dehydrated in ethanol (0 C), transferred gradually to propylene oxide (0 C), and infiltrated with Araldite at room temperature. The cells were then transferred to fresh resin in Beem capsules, soaked for 2 days, and baked at 60 C for 40 hr.

Sections were cut with a diamond knife, stained with both uranyl acetate and lead citrate, mounted on uncoated grids, and examined in a Philips 300 electron microscope.

RESULTS

General Characteristics of Binding Assay. Figure 1 shows that adherence of bacteria to *Datura* cells gradually increased during the first 60 min of incubation and reached a maximum binding after 2 hr. Figure 2 shows that bacteria adherence to *Datura* cells increased linearly with increasing number of bacteria added up to 5×10^8 cells/ml of binding mixture. Approximately 10% of the bacteria were bound to the *Datura* cells. The curve suggests that at the higher density level (5×10^9 cells/ml) binding is approaching saturation.

Effect of Medium Changes on Binding. Binding experiments were performed at various pH values using 10 mM MES buffer (pH 5.0–6.0), 10 mM Tris-HCl (pH 6.1–7.8), or 10 mM K-phosphate (pH 4.5–8.9). A very distinct maximum binding of bacteria to *Datura* cells was observed at pH 6.0 (Fig. 3). At pH higher than 7.0 there was a significant reduction in bacteria-plant cell adherence.

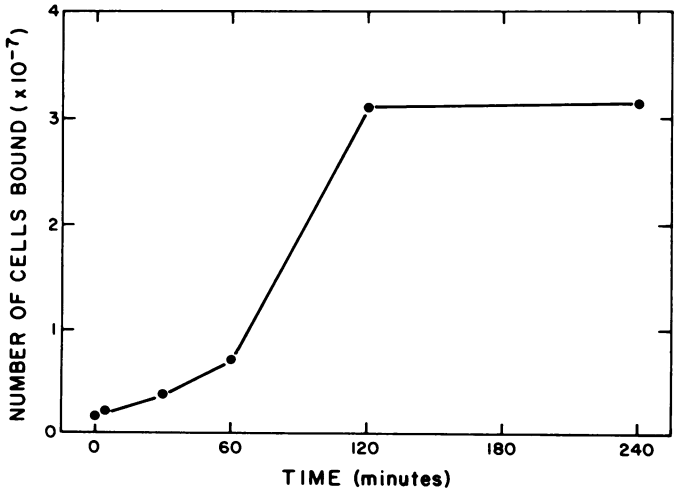

FIG. 1. Kinetics of *A. tumefaciens* strain B6 binding to *Datura* cells. Binding experiments were carried out at 28 C in standard binding mixture with ^{32}P-labeled virulent *A. tumefaciens* strain B6 (2.1×10^8 cells, 2.6×10^5 cpm). Numbers are average of duplicate experiments and expressed as number of bacteria bound per mg dry weight of *Datura* cells (maximum standard deviation, 9.9%).

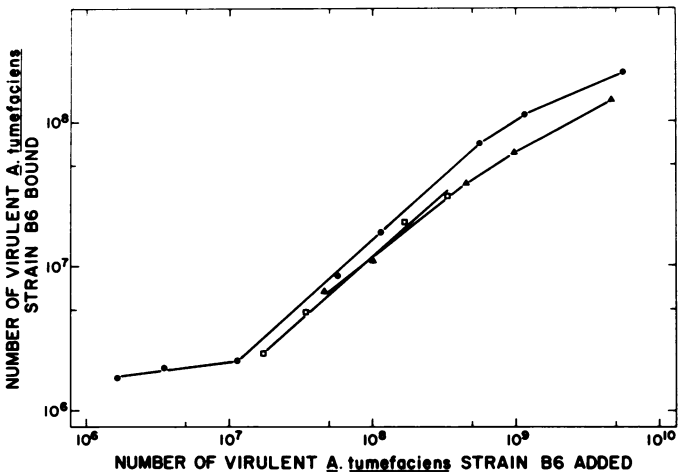

FIG. 2. Relationship between numbers of virulent *A. tumefaciens* strain B6 added to standard binding mixture and numbers of virulent *A. tumefaciens* strain B6 bound to *Datura* cells. Figure shows results of three experiments (three different preparations of the bacteria and *Datura* cells). Data are expressed as number of bacteria bound per mg dry weight of *Datura* cells in standard binding mixture (maximum standard deviation, 18.9%).

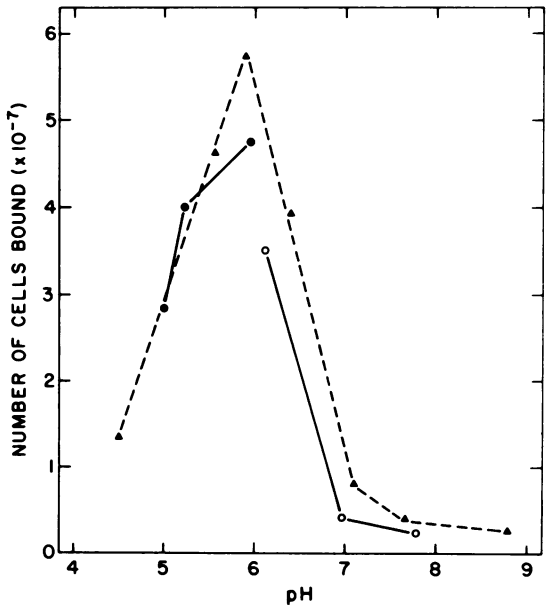

FIG. 3. Effect of pH on the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells. Binding experiments were carried out at 28 C for 2 hr in standard binding mixture with virulent *A. tumefaciens* strain B6 (2.7×10^8 cells, 1.9×10^5 cpm). Data are expressed as number of bacteria bound per mg dry weight of *Datura* cells (maximum standard deviation, 21.4%). (▲---▲): 10 mM K-phosphate; (●——●): 10 mM MES; (○——○): 10 mM Tris-HCl.

As shown in Table I, the presence of Ca^{2+} in the binding mixture markedly inhibited the binding. Mg^{2+} caused a slight reduction in binding at a concentration of 10 mM while EDTA had little effect over the concentration range of 0.1 to 10.0 mM.

The data in Table II show bacteria binding to *Datura* cells to be a temperature-dependent phenomenon. Maximum binding occurred at 28 C. At 0, 13, 45, or 60 C a little binding occurred.

It is well known that virulent *A. tumefaciens* strain B6 can utilize octopine as a nitrogen source (5) and that crown gall tumor cells induced by strain B6 are able to synthesize octopine (12). Octopine and its analogs were tested to evaluate the effect of the compounds on the binding of bacteria to *Datura* cells. As shown in Table III, canavanine was slightly inhibitory but octopine had no effect. Arginine at the concentration of 1.0 mM was found to enhance the

Table I. Effect of Ca^{++}, Mg^{++}, and EDTA on the binding of A. tumefaciens to Datura cells from suspension culture.[1]

Concentration (mM)		number of cells bound[2] (x 10^{-7})
None		4.44 ± 0.16
Ca^{++}	0.1	3.76 ± 0.26
	1.0	3.54 ± 0.00
	10.0	1.89 ± 0.30
Mg^{++}	0.1	4.39 ± 0.13
	1.0	4.01 ± 0.35
	10.0	3.37 ± 0.15
EDTA	0.1	4.07 ± 0.35
	1.0	4.12 ± 0.23
	10.0	3.79 ± 0.20

[1]Binding experiments were carried out at 28 C for 2 hr in standard binding mixture with ^{32}P-labeled virulent A. tumefaciens strain B6 (3.7 x 10^8 cells, 3.6 x 10^5 cpm).

[2]Numbers are average of duplicate experiments, expressed as number of cells bound per mg dry weight of Datura cells.

Table II. Effect of incubation temperature on the binding of A. tumefaciens to Datura cells from suspension culture.[1]

Temperature (C)	number of cells bound[2] (x 10^{-6})
0	0.75 ± 0.16
13	0.60 ± 0.03
24	1.22 ± 0.05
28	1.55 ± 0.16
35	1.35 ± 0.05
47	0.09 ± 0.02
60	0.11 ± 0.01

[1]Binding experiments were carried out for 2 hr in standard binding mixture with ^{32}P-labeled virulent A. tumefaciens strain B6 (3.0 x 10^7 cells, 5.0 x 10^4 cpm).

[2]Numbers are average of duplicate experiments, expressed as number of cells bound per mg dry weight of Datura cells.

binding up to 40% above the control.

Addition of protease (Sigma) slightly increased the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells. DNase (Worthington), RNase (Worthington), and lipase (Sigma) had little effect on the adherence. Phosphatidylcholines such as lecithin (Sigma) and lysolecithin (Sigma) slightly enhanced the binding, but concanavalin A (Sigma) and soybean lectin (Sigma) showed no stimulation of the binding. Poly-L-lysine more than doubled binding (Table IV).

Bacterial Specificity. In order to test that the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells is specific for this microorganism, binding competition experiments using other microorganisms were performed. Table V shows the effect of a 2-hr preincubation or simultaneous addition of other microorganisms on the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells. Simultaneous incubation of virulent *A. tumefaciens* strain B6 with other microorganisms had little effect on the binding. With the exception of *Micrococcus lysodeikticus*, preincubation of plant cells with the other microorganisms resulted in some competition with virulent *A. tumefaciens* strain B6 for binding. Related strains *A. tumefaciens* A6 (virulent) and 5Gly-Fe (avirulent) had considerable increase of strain B6 binding to *Datura* cells while heat-killed strain IIBNV6 (avirulent) had little effect on the binding when precultured. We found that the adherence of virulent *A. tumefaciens* strain B6 to *Datura* cells was stimulated by approximately 40% when simultaneously incubated with avirulent *A. tumefaciens* strain IIBNV6 (8 × 10^8 cells/ml) (Fig. 4). Preincubation of *Datura* cells with avirulent *A. tumefaciens* strain IIBNV6 caused a slight inhibition of the adherence of virulent *A. tumefaciens* strain B6. Addition of nonradioactive virulent *A. tumefaciens* strain B6 to radioactive virulent *A. tumefaciens* strain B6 as expected decreased the amount of radioactivity bound to *Datura* cells (data not shown).

Plant Cell Specificity. To determine the relative binding ability of strain B6 to various plant cells, binding experiments were performed by using cell suspension cultures of dicotyledons, monocotyledons, and liverworts (Table VI). The value obtained with *Datura* cells was used as standard. Cells of vetch, pea and particularly soybean had a significantly higher B6-binding capacity than *Datura* cells. The cells of corn, sorghum, rice, and wheat had much lower binding affinity. The binding of strain B6 to the liverwort (*Marchantia polymorpha,* supplied by K. Ono, Kuma-

Table III. Effect of octopine, canavanine, and arginine on the binding of A. tumefaciens to Datura cells from suspension culture.[1]

Additive (mM)		number of cells bound[2] (x 10^{-7})
None		2.92 ± 0.15
Octopine	0.1	2.74 ± 0.04
	1.0	2.89 ± 0.02
	10.0	2.90 ± 0.02
Canavanine	0.1	2.64 ± 0.04
	1.0	2.50 ± 0.06
	10.0	1.80 ± 0.22
Arginine	0.01	2.82 ± 0.07
	0.1	3.79 ± 0.05
	1.0	4.10 ± 0.23
	10.0	3.77 ± 0.21

[1]Binding experiments were carried out at 28 C for 2 hr in standard binding mixture. The indicated concentrations of compounds were added 60 min prior to incubation with ^{32}P-labeled virulent A. tumefaciens strain B6 (2.5 x 10^8 cells, 2.5 x 10^5 cpm).

[2]Numbers are average of duplicate experiments, expressed as number of cells bound per mg dry weight of Datura cells.

Table IV. Effect of various compounds on the binding of virulent A. tumefaciens strain B6 to Datura cells from suspension culture.[1]

Additive (50μg/ml)	Number of cell bound[2] (x 10^{-6})
None	2.28 ± 0.34
Protease	3.40 ± 0.11
DNase	1.92 ± 0.02
RNase	2.32 ± 0.04
Lipase	2.30 ± 0.13
Lecithin	2.68 ± 0.09
Lysolecithin	3.14 ± 0.24
Concanavalin A.	2.52 ± 0.40
Lectin, soybean	2.02 ± 0.09
Poly-L-lysine	6.15 ± 0.48

[1]Binding experiments were carried out at 28 C for 2 hr in standard binding mixture. The indicated compounds were added 60 min prior to incubation with ^{32}P-labeled virulent A. tumefaciens strain B6 (2.0 x 10^7 cells, 2.2 x 10^4 cpm).

[2]Numbers are average of duplicate experiments, expressed as number of cells bound per mg dry weight of Datura cells.

Table V. Effect of related and non-related microorganisms on the binding of virulent A. tumefaciens strain B6 to Datura cells from suspension culture.[1]

Microorganisms	number of cells bound[2] (x 10^{-7})	
	simultaneous incubation	preincubation
None	2.15 ± 0.03	2.31 ± 0.37
A. tumefaciens A6	3.04 ± 0.07	3.25 ± 0.65
A. tumefaciens 5Gly-Fe	2.43 ± 0.10	2.71 ± 0.38
A. tumefaciens IIBNV6 (heat-killed)	2.44 ± 0.08	2.29 ± 0.16
M. lysodeikticus[3]	2.20 ± 0.03	2.42 ± 0.06
E. coli[3]	2.31 ± 0.20	2.01 ± 0.01
S. typhimurium[3]	2.37 ± 0.02	1.98 ± 0.01
R. japonicum	2.09 ± 0.09	1.79 ± 0.25

[1]Binding experiments were carried out at 28 C for 2 hr in the standard binding mixture. The indicated bacteria, virulent A. tumefaciens A6 (3.6 x 10^9 cells), avirulent A. tumefaciens 5Gly-Fe (2.0 x 10^9 cells), avirulent A. tumefaciens IIBNV6 (3.6 x 10^9 cells, heat-killed, 80 C 15 min), M. lysodeikticus (2.1 x 10^7 cells), E coli (7.4 x 10^7 cells), S. typhimurium (8.3 x 10^7 cells), and R. japonicum (2.0 x 10^9 cells) were added simultaneously or added 2 hr prior to addition of ^{32}P-labeled virulent A. tumefaciens B6 (1.4 - 2.3 x 10^8 cells, 3.6 - 6.5 x 10^5 cpm).

[2]Numbers are average of duplicate experiments, expressed as number of cells bound per mg dry weight of Datura cells.

[3]Higher than 10^9 bacterial cells caused extensive damage to Datura cells during the incubation (data not shown).

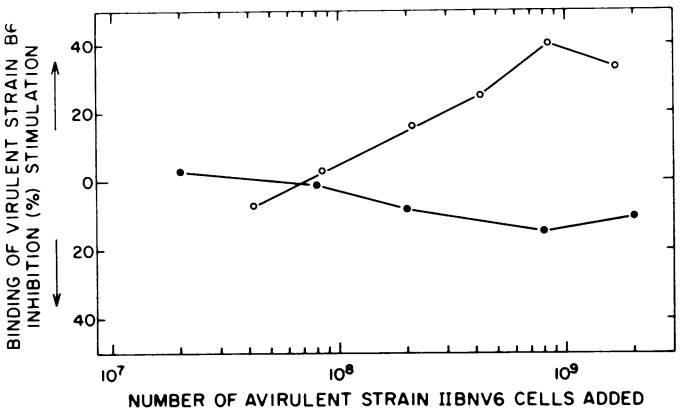

FIG. 4. Effect of avirulent *A. tumefaciens* strain IIBNV6 on the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells. Data are expressed as percentage of stimulation and inhibition of *A. tumefaciens* strain B6 binding to *Datura* cells by addition of indicated numbers of avirulent *A. tumefaciens* IIBNV6 strain (maximum standard deviation 11.9%). (○——○): Simultaneous incubation (2 hr) of strain IIBNV6 with strain B6 (3.0 × 10^7 cells, 2.2 × 10^4 cpm); (●——●): preincubation (2 hr) of strain IIBNV6 with *Datura* cells and additional incubation (2 hr) with strain B6 (3.0 × 10^7 cells, 2.2 × 10^4 cpm).

moto, Japan) was similar to that obtained with *Datura* cells.

Microscopic Examination of Adherence. In order to observe visually the location of the binding sites of virulent *A. tumefaciens* strain B6 to *Datura* cells, autoradiography was performed by using [^{14}C]adenine-labeled strain B6 cells. Figure 5, A and B, shows that bacterial cells were bound predominantly to the cell walls. The clustering of grains indicates that the bacteria may preferentially bind to specific areas of the cell wall. Figure 5C shows such a clustering phenomenon over a single cell in a three-cell aggregate.

Results of the electron microscope study indicate that virulent *A. tumefaciens* strain B6 cells are tightly bound to the cell walls of *Datura* cells (Fig. 6, A and E). The attached bacteria were often localized in the intercellular spaces (Fig. 6, A–C). Most bacteria were associated with fine fibrillar material on the surface of the *Datura* cells. In the intercellular spaces these fibrils were commonly present as a compact network (Fig. 6, A and C). Loose aggregates of fibrils were associated with bacteria elsewhere on the *Datura* cells (Fig. 6D). Occasionally unbound bacteria appeared to be joined to the plant cell walls by these fibrils (Fig. 6D).

Table VI. Binding of virulent A. tumefaciens strain B6 to various plant cells from suspension culture.[1]

Plant species	number of cells bound[2] (x 10^{-6})	%
Datura innoxia[4]	2.95 ± 0.10	100
Daucus carota (carrot)[3]	3.14 ± 0.11	106
Haplopappus gracilis[3]	4.10 ± 0.29	139
Lactuca sativa (lettuce)[6]	3.86 ± 0.10	131
Brassica napus (rapeseed)[5]	2.36 ± 0.21	80
Nicotiana suaveolens (tobacco)[3]	2.16 ± 0.02	73
Glycine max (soybean)[3]	8.64 ± 0.13	293
Pisum sativum (pea)[3]	6.47 ± 0.28	219
Vicia hajastana (vetch)[5]	5.89 ± 1.04	200
Sorghum bicolor (sorghum)[4]	1.64 ± 0.08	56
Triticum monococcum (wheat)[3]	1.45 ± 0.28	49
Oryza sativa (rice)[3]	1.93 ± 0.05	65
Zea mays (corn)[4]	2.50 ± 0.14	85
Bromus inermis (bromegrass)[5]	2.80 ± 0.00	95
Marchantia polymorpha (liverwort)[4]	2.84 ± 0.26	96

[1]Binding experiments were carried out at 28 C for 2 hr in standard binding mixture. Prior to incubation with ^{32}P-labeled virulent A. tumefaciens strain B6 (3.6 x 10^7 cells, 3.0 x 10^4 cpm), plant cells were washed with 1-B5 medium and used.

[2]Numbers are average of duplicate experiments, expressed as number of cells bound per mg dry weight of plant cells.

[3]Plant cells were cultured in 1-B5 or 1-B5C medium (28).

[4]Plant cells were cultured in 1-M51C medium.

[5]Plant cells were cultured in ER medium (10).

[6]Plant cells were cultured in MS medium (26).

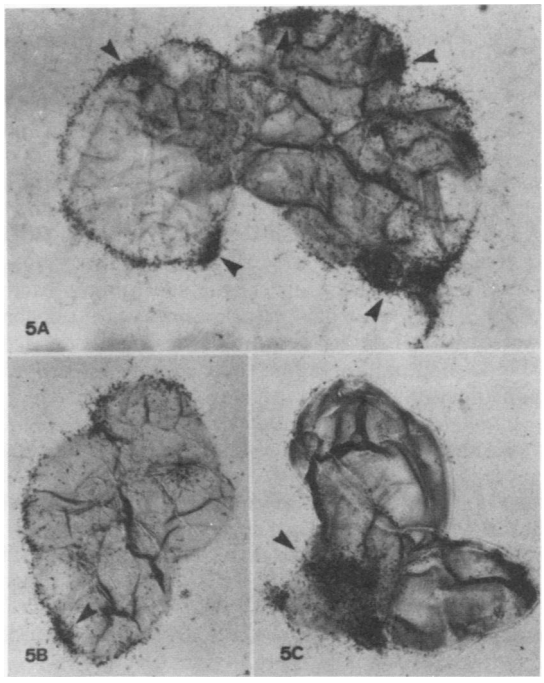

FIG. 5. A and B: autoradiographs of [^{14}C]adenine-labeled *A. tumefaciens* strain B6 (specific radioactivity, 0.9 × 10^{-3} dpm/cell) adhering to *Datura* cell walls. Note greater binding at certain areas of cells (arrows). C: autoradiograph showing the bacteria binding to a single cell in a three-cell cluster (arrow) (× 2,400).

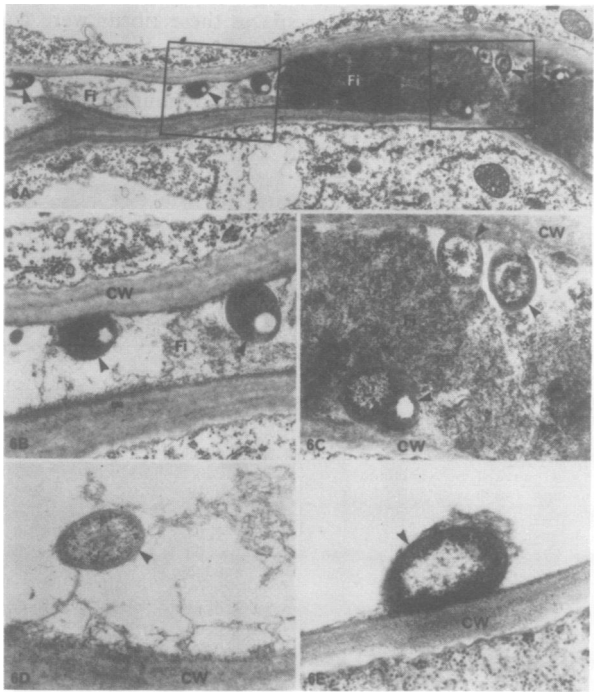

FIG. 6. A: electron micrograph showing an intercellular space between *Datura* cells. *A. tumefaciens* cells (arrows) are tightly bound to the plant cell walls and are surrounded by a network of fine fibrils (Fi). The areas outlined in black are shown in B and C (× 11,200). B: enlargement from A showing *A. tumefaciens* cells (arrows) tightly bound to a cell wall (CW) of *Datura* cells. Note the fine fibrils (Fi) surrounding the bacteria (× 32,500). C: enlargement from A showing *A. tumefaciens* cells (arrows) tightly bound to the cell wall (CW) of *Datura* cells. A very compact network of fine fibrils (Fi) surrounds the bacteria (× 35,000). D: electron micrograph showing an *A. tumefaciens* cell (arrow) connected to the *Datura* cell wall (CW) by fibrillar material (× 36,000). E: electron micrograph showing an *A. tumefaciens* cell (arrow) bound tightly to a *Datura* cell wall (CW) and covered with fibrillar material (× 55,000).

DISCUSSION

In vitro binding of virulent *A. tumefaciens* strain B6 to *Datura* cells reached a maximum level after 2-hr incubation. Yajko and Hegeman (36) reported similar kinetics of association of ^{3}H-labeled *A. tumefaciens* with carrot root discs. They reported that maximum binding required a 2-hr incubation at which time 4% of the input radioactivity was associated with the discs. Yajko and Hegeman (36) suggested that the association of the radioactivity with the discs resulted from transfer of DNA from the bacteria to the carrot cells. In the case of *A. tumefaciens-Datura* cells binding approximately 10% of the input radioactivity was associated with *Datura* cells. Our autoradiographic and electron microscopic analyses demonstrated that bacteria were associated with cells.

In the *A. tumefaciens-Datura* cell system the number of bacteria bound was a linear function of the input number of bacteria over the range of 1.1×10^7 to 5×10^8 cells/ml. Lippincott and Lippincott (15) reported a bioassay system using pinto bean leaves and found a linear relationship between the number of tumors per leaf and the number of virulent *A. tumefaciens* strain B6 applied up to approximately 10^9 cells/ml. Bogers (3) observed a linear relationship between the mean tumor weight and the number of inoculated strain B6 from 10^3 to 10^7 cells/ml on *Kalanchoë daigremontiana* stems.

The induction of crown gall tumors is thermosensitive. Braun reported that *K. daigremontiana* infected with *A. tumefaciens* formed tumors at 25 C but not 32 C (6). In our binding experiments, little binding was detected at 0, 13, 45, or 60 C, indicating that binding occurs only at a physiological temperature.

A unique characteristic of crown gall tumor cells is their production of unusual amino acids: octopine [N^2-(D-1-carboxyethyl)-L-arginine] (12), or nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine] (25). The bacterial strains rather than plant hosts determine whether octopine or nopaline is synthesized by the tumor cells (5). Lippincott and Lippincott (22) reported on the promotion of crown gall tumor growth by octopine or nopaline. Petit *et al.* (29) reported that octopine induced conjugative activity of *A. tumefaciens* Ti plasmids. Our observations on the effects of octopine, arginine which is the substrate for octopine dehydrogenase in tumor cells (4), and canavanine which is also a substrate of the enzyme (4) showed some interesting effects on the binding of virulent *A. tumefaciens* strain B6 to *Datura* cells. Further studies are required to determine the mechanism of these compounds on the binding of bacteria to plant cells.

Addition of DNase, RNase, or lipase to the binding mixture had no effect on the bacteria-plant cell association. Yajko and Hegeman (36) reported that addition of 4 mg/ml DNase to the incubation mixture had no effect on the amount of radioactivity retained by the tissue. Braun and Wood (8) reported that the treatment of the *K. daigremontiana* plant wound site with RNase inhibited the tumor induction. Nevins *et al.* (27) demonstrated pronounced alterations of some properties of the bacterial cells by treatment with RNase.

Anand *et al.* (1) reported that concanavalin A and soybean lectin may enhance tumor induction by facilitating the attachment of the bacteria to the potential tumor cells. However, concanavalin A and soybean lectin had no effect on our bacteria-*Datura* cell adherence system. Lecithin and lysolecithin enhanced the bacteria-*Datura* cell binding. There are few reports on the influence of these compounds on tumor induction *in vivo* (19), although *A. tumefaciens* was found to contain a relatively large amount of phosphatidylcholine in their cell envelopes (23). Polycations such as poly-L-ornithine, poly-L-lysine, and DEAE-dextran are known to increase greatly the uptake of plant virus (32), its RNA (2), and DNA (28) by plant materials. In our binding experiments, enhanced binding of bacteria by poly-L-lysine could result from bacteria aggregation which would cause increased attachment to plant cells.

In the *Datura*-binding system related and nonrelated bacteria did not compete with virulent *A. tumefaciens* strain B6 suggesting that a specific attachment site was involved. Lippincott and Lippincott (15, 16) reported that viable or heat-killed cells only of closely related bacteria reduced the number of tumors when mixed into inoculum. They tested avirulent *A. tumefaciens* strain IIBNV6, avirulent strain 5 Gly-Fe, and nonrelated bacteria such as *Escherichia coli, Bacillus megaterium,* and *Rhizobium meliloti* against virulent *A. tumefaciens* strain B6. Yajko and Hegeman (36) also observed a site specificity for *A. tumefaciens* binding to carrot root discs using *E. coli* as a control.

Avirulent *A. tumefaciens* strain IIBNV6 has been reported to promote tumor initiation when inoculated together with virulent *A. tumefaciens* strain B6 (16). Inoculation of the tumorigenic strain on leaves previously inoculated with avirulent *A. tumefaciens* strain IIBNV6 showed loss of the capacity to complement. We have observed quite a similar situation in the *A. tumefaciens-Datura* cell-binding experiments. This suggests that binding enhancement by avirulent *A. tumefaciens* strain IIBNV6 may occur in the early attachment stage of the process because preincubation of strain IIBNV6 with *Datura* cells did not enhance the binding. Lippincott *et al.* (16, 21) proposed that bacterial genes or gene products may be involved in the complementation of tumor initiation by avirulent strains of agrobacteria.

Recently Lippincott *et al.* (17, 20) reported that cell walls from susceptible pinto bean leaves exhibited the characteristics expected of the host adherence site. They also demonstrated that crown gall initiation by *A. tumefaciens* is inhibited by cell walls from dicotyledonous plants, but not by cell walls from monocotyledonous

plants. Although we used plant cells from suspension cultures for bacteria-binding experiments, our observation that strain B6 adheres in greater numbers to dicotyledonous cells than to monocotyledonous cells is consistent with the observations reported by Lippincott and Lippincott (20). Legume plant cells tested exhibited an even higher affinity for strain B6, suggesting that these cells may possess a greater number of attachment sites per unit dry weight.

Both autoradiography and electron microscopy clearly illustrate the binding of virulent *A. tumefaciens* strain B6 to cell wall of *Datura* cells. Bogers (3) reported a similar association between *A. tumefaciens* and stem cells from infected *K. daigremontiana* plants and suggested that specific binding sites were involved. A recent scanning electron microscope study has indicated that *A. tumefaciens* will also bind to cell walls of moss protonema (31). The mechanism of binding of *A. tumefaciens* to cell walls of plants is unknown at present. The bacteria were commonly associated with fibrils on the surface of *Datura* cells (Fig. 6D) and it is possible that these fibrils are involved in the binding process. Similar fibrils have been described on the surface of cultured cells of *Daucus, Ipomoea,* and *Phaseolus* (14) as well as on cells of roots (13) and leaves (30). Extracellular polysaccharides produced on the surface of *A. tumefaciens* (9, 35) may specifically interact with the fibrils on *Datura* cells to facilitate binding.

We have observed that *A. tumefaciens* adheres to plant cells from suspension culture. Matthysse *et al.* (24) briefly reported the interaction between *A. tumefaciens* and tobacco tissue-cultured cells. Although we have not succeeded in inducing tumors in suspension cultures, the *in vitro* binding system employing *A. tumefaciens* and plant cells is a promising technique for investigating the mechanism of tumor induction by *A. tumefaciens.*

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LITERATURE CITED

1. Anand VK, SG Pueppke, GT Heberlein 1977 The effect of lectins on *Agrobacterium tumefaciens* caused crown gall tumor induction. Plant Physiol 59: S-109
2. Aoki S, I Takebe 1969 Infection of tobacco mesophyll protoplasts by tobacco mosaic virus ribonucleic acid. Virology 39: 439–448
3. Bogers RJ 1972 On the interaction of *Agrobacterium tumefaciens* with cells of *Kalanchoea daigremontiana.* Proceedings of the Third International Conference on Plant Pathogenic Bacteria, Wageningen, The Netherlands, pp 239–250
4. Bomhoff GH 1974 Studies on crown gall—a plant tumor: investigations on protein composition and on the use of guanidine compounds as a marker for transformed cells. PhD thesis. University of Leyden, The Netherlands
5. Bomhoff G, PM Klapwijk, HCM Kester, RA Schilperoort, JP Hernalsteens, J Schell 1976 Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens.* Mol Gen Genet 145: 177–181
6. Braun AC 1947 Thermal studies on the factors responsible for tumor initiation in crown gall. Am J Bot 34: 234–240
7. Braun AC, RJ Mandle 1948 Studies on the interaction of the tumor-inducing principle in crown gall. Growth 12: 255–269
8. Braun AC, HN Wood 1966 On the inhibition of tumor inception in the crown-gall disease with the use of ribonuclease A. Proc Nat Acad Sci USA 56: 1417–1422
9. Cagle GD 1975 Fine structure and distribution of extracellular polymer surrounding selected aerobic bacteria. Can J Microbiol 21: 395–408
10. Eriksson T 1965 Studies on the growth requirements and growth measurements of cell cultures of *Haplopappus gracilis.* Physiol Plant 18: 976–993
11. Fowke LC 1975 Electron microscopy of protoplasts. *In* OL Gamborg, LR Wetter, eds, Plant Tissue Culture Methods. National Research Council of Canada, Saskatoon, pp 55–59
12. Goldmann AD, W Thomas, G Morel 1969 Sur la structure de la nopaline metabolite anormal de certaines tumeurs de crown-gall. CR Acad Sci 268: 852–854
13. Leppard GG, JR Calvin 1972 Electron-opaque fibrils and granules in and between the cell walls of higher plants. J Cell Biol 53: 695–703
14. Leppard GG, JR Calvin, J Rose, SM Martin 1971 Lignofibrils on the external cell wall surface of cultured plant cells. J Cell Biol 50: 63–80
15. Lippincott BB, JA Lippincott 1969 Bacterial attachment to a specific wound site as an essential stage in tumor initiation by *Agrobacterium tumefaciens.* J Bacteriol 97: 620–628
16. Lippincott BB, JB Margot, JA Lippincott 1977 Plasmid content and tumor initiation complementation by *Agrobacterium tumefaciens* IIBNV6. J Bacteriol 132: 824–831
17. Lippincott BB, MH Whatley, JA Lippincott 1977 Tumor induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. Plant Physiol 59: 388–390
18. Lippincott JA, BB Lippincott 1975 The genus *Agrobacterium* and plant tumorigenesis. Annu Rev Microbiol 29: 377–405
19. Lippincott, JA, BB Lippincott 1977 Nature and specificity of the bacterium-host attachment in *Agrobacterium* infection. *In* B Solheim, J Raa, eds, Cell Wall Biochemistry Related to Specificity in Host-plant Pathogen Interactions. Norway Universitetsforlaget, Oslo, pp 439–451
20. Lippincott JA, BB Lippincott 1978 Cell walls of crown-gall tumors and embryonic plant tissues lack *Agrobacterium* adherence sites. Science 199: 1075–1078
21. Lippincott JA, BB Lippincott 1978 Tumor initiation complementation on bean leaves by mixtures of tumorigenic and nontumorigenic *Agrobacterium rhizogenes.* Phytopathology 68: 365–370
22. Lippincott JA, BB Lippincott, C-C Chang 1972 Promotion of crown-gall tumor growth by lysopine, octopine, nopaline, and carnosine. Plant Physiol 49: 131–137
23. Manasse RJ, WA Corpe 1967 Chemical composition of cell envelopes from *Agrobacterium tumefaciens.* Can J Microbiol 13: 1591–1603
24. Matthysse AG, PM Wyman, HL Miller 1977 Interaction between *Agrobacterium tumefaciens* and tobacco tissue culture cells. Plant Physiol 59: S-108
25. Menage A, G Morel 1964 Sur la presence d'octopine dans les tissus de crown-gall. CR Acad Sci 259: 4795–4796
26. Murashige T, F Skoog 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473–497
27. Nevins MP, DW Grant, RR Baker 1970 Ribonuclease-induced alterations in *Agrobacterium tumefaciens.* Phytopathology 60: 381–382
28. Ohyama K, OL Gamborg, RA Miller 1972 Uptake of exogenous DNA by plant protoplasts. Can J Bot 50: 2077–2080
29. Petit A, J Tempe, A Kerr, M Holsters, M van Montagu, J Schell 1978 Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature 271: 570–572
30. Politis DJ, RN Goodman 1978 Localized cell wall appositions: incompatibility response of tobacco leaf cells to *Pseudomonas pisi.* Phytopathology 68: 309–316
31. Spiess LD, JC Turner, PG Mahlberg, BB Lippincott, JA Lippincott 1977 Adherence of *Agrobacteria* to moss protonema and gametophores viewed by scanning electron microscopy. Am J Bot 64: 1200–1208
32. Takebe I, Y Otsuki 1969 Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. Proc Nat Acad Sci USA 64: 843–848
33. Van Larebeke N, G Engler, M Holsters, S Van den Elsacker, I Zaenen, RA Schilperoort, J Schell 1974 Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature 252: 169–170
34. van Larebeke N, C Genetello, J Schell, RA Schilperoort, AK Hermans, JP Hernalsteens, M van Montagu 1975 Acquisition of tumor-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature 255: 742–743
35. Whatley MH, JS Bodwin, BB Lippincott, JA Lippincott 1976 Role of *Agrobacterium* cell envelope lipopolysaccharide in infection site attachment. Infect Immun 13: 1080–1083
36. Yajko DM, GD Hegeman 1971 Tumor induction by *Agrobacterium tumefaciens*: specific transfer of bacterial deoxyribonucleic acid to plant tissue. J Bacteriol 108: 973–979
37. Zaenen I, N van Larebeke, H Teuchy, M van Montagu, J Schell 1974 Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J Mol Biol 86: 109–127