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Tumor Induction by *Agrobacterium* Involves Attachment of the Bacterium to a Site on the Host Plant Cell Wall[1]

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BARBARA B. LIPPINCOTT, MARIAMNE H. WHATLEY, AND JAMES A. LIPPINCOTT
Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201

ABSTRACT

Cell wall preparations from primary bean leaves were found to inhibit tumor initiation by *Agrobacterium tumefaciens* strain B6 when inoculated with the bacteria on bean leaves. Membrane fractions from these same leaves were noninhibitory. The cell walls were effective when applied prior to or with bacteria, but application of cell walls about 15 minutes after bacteria did not affect the number of tumors initiated. Much of the inhibitory activity of the plant cell walls was eliminated by pretreatment with dead site-attaching bacteria or with lipopolysaccharide from these bacteria. Cells and lipopolysaccharide from non-site-attaching agrobacteria had no effect on the activity of the plant cell walls. About 30% inhibition of tumor initiation was obtained with plant cell walls at 50 μg/ml dry weight, and at 10 mg/ml dry weight about 70% inhibition was typical. Both early and late appearing tumors were affected by the cell walls, indicating that they do not exclusively affect tumors arising from either small or large wounds. These data show that plant cell walls but not membranes contain surfaces to which *A. tumefaciens* adheres and these exhibit the specificity typical of the host site to which virulent agrobacteria must attach to induce tumors. It is concluded that some portion of wound-exposed plant cell wall constitutes the host adherence site in *Agrobacterium* infections.

Competition experiments in which dead or avirulent agrobacteria are inoculated with viable tumorigenic bacteria have shown that attachment of *Agrobacterium tumefaciens* to a wound-exposed host site is essential for crown gall tumor induction (1, 4, 6, 10, 12). Although this attachment clearly exhibits considerable specificity on the part of the bacterium (6), no evidence has been obtained to indicate the nature of the plant component of attachment or its specificity. Elsewhere, we have reported that a LPS[2] present in the outer membrane of *Agrobacterium* is involved in site attachment (13). This paper demonstrates that host cell wall preparations exhibit the predicted characteristics of the plant component of the *Agrobacterium* host site attachment process which is the first event in the tumor induction process.

MATERIALS AND METHODS

Bacterial Strains and Infectivity Tests. Cultures of *A. tumefaciens* (Smith and Town.) Conn, strains B6 and IIBNV6, and *Agrobacterium radiobacter* (Beijerinck and van Delden) Conn, strains ATCC 6467 and S1005, were grown to stationary phase (48 hr), centrifuged, and suspended in 0.05 M K-phosphate (pH 7) as previously described (6). Viable cell counts were determined by dilution and plating. Infectivity was assayed on primary leaves of 7 day old *Phaseolus vulgaris* L. var. 'Pinto' seedlings using the carborundum wounding procedure described by Lippincott and Heberlein (7). In this assay, the mean number of tumors/leaf is proportional to the number of virulent bacteria in the inoculum and the standard error is about ±20% of the mean number of tumors/leaf (5, 7).

Preparation of Bacterial Lipopolysaccharide. Bacterial LPS was obtained from 48-hr cultures of agrobacteria as described by Whatley *et al.* (13). The cells were harvested by centrifugation and homogenized in a Braun homogenizer with glass beads. After removing whole cells by centrifugation, LPS was isolated by a phenol treatment. The LPS was dialyzed, lyophilized, and resuspended in water. It was mixed with plant cell walls in this form. In some experiments, the LPS was further purified by ultracentrifugation and passage through a column of Sepharose 2B.

Plant Cell Wall Preparations. Cell walls were prepared from the primary leaves of 7-day-old Pinto bean plants by a modification of the method of Nevins *et al.* (2, 11). The leaves were homogenized in a Waring Blendor in 0.5 M K-phosphate (pH 7), then centrifuged 15 min at 2000*g*. The precipitate was washed three times with the above buffer, then suspended in acetone and again homogenized in a Waring Blendor. The homogenate was filtered through Whatman No. 1 filter paper with the aid of an aspirator and further washed three times with acetone. The precipitate was air-dried at room temperature overnight, weighed, and stored in a desiccator at room temperature. The yield of dry cell walls was approximately 1 g/10 g of leaves, wet weight (approximately 100 leaves).

Plant Cell Membrane Preparations. Cell membranes were prepared from the primary leaves of 7-day-old pinto beans by a modification of the method of Hodges and Leonard (3). Leaves were homogenized in 25 mM tris-MES buffer (pH 7.2) containing 0.25 M sucrose and 3 mM EDTA. After straining through cheesecloth, the homogenate was centrifuged for 30 min at 5000*g*. The supernatant was then centrifuged for 15 min at 13,000*g*. The precipitate was suspended in the above buffer and centrifuged again in the same manner to yield the 13,000*g* membrane fraction. The supernatant from the first 13,000*g* centrifugation was centrifuged for 30 min at 80,000*g*. The precipitate from this centrifugation, after washing in the same manner, was called the 80,000g fraction. For infectivity tests, the membrane fractions were combined with *A. tumefaciens* strain B6 which had been suspended in the above tris-MES buffer. The control B6 was also in the same buffer.

Treatment of Cell Walls with Avirulent Bacteria or Lipopolysaccharide. Pinto cell walls were suspended in 0.05 M K-phosphate (pH 7) at a concentration of 20 mg/ml. One ml of this suspension was mixed with 1 ml of either *A. tumefaciens* strain IIBNV6 (approximately 10^{10} cells/ml in the above phosphate

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[2] Abbreviation: LPS: lipopolysaccharide.

buffer), or with 400 μg/ml of bacterial LPS. The mixture was allowed to stand at room temperature for 15 min, then was centrifuged 15 min at 2000*g*. The precipitate was washed once with 2 ml of phosphate buffer, then resuspended in buffer to a total volume of 1 ml. One ml of *A. tumefaciens* strain B6 in phosphate buffer was added, the samples mixed on a Vortex mixer and inoculated on Pinto bean leaves.

RESULTS

Initial considerations suggested that either plant cell membranes or cell walls might contain the site to which *Agrobacterium* attaches in the process of tumor initiation. As shown in Table I, membrane-rich fractions isolated from bean leaves of optimum susceptibility to *A. tumefaciens* had no significant effect on the number of tumors obtained when mixed and inoculated with tumorigenic bacteria on similar bean leaves. If the plant site attachment component were present, a portion of the bacteria in the inoculum should adhere to these preparations, thus preventing them from attaching to wound-exposed host sites and inducing tumors.

Bean leaf cell wall preparations, unlike the membrane fractions, markedly reduced tumor induction when mixed and inoculated with *A. tumefaciens* (Tables I and II). As further shown, this inhibition occurs in mixtures or when the cell wall preparation is applied to wounded leaves about 15 min prior to the bacterium. Applying the bacterium first and the cell walls about 15 min later eliminates the inhibitory activity of the cell walls, consistent with the supposition that they act through a reduction in the number of bacteria which remain free to attach to potential tumor sites. Since bacterial attachment is largely complete on bean leaves within 15 min (6), cell walls would not be expected to inhibit at this time if their activity depends only on their competition for bacteria prior to their attachment.

Figure 1 illustrates the effects of various concentrations of bean leaf cell walls on tumor induction by strain B6. Significant inhibition was obtained with concentrations of cell wall below 0.1 mg/ml. Cellulose suspensions obtained by homogenizing Whatman filter paper in a Waring Blendor were noninhibitory at 10 mg dry weight/ml, indicating this inhibition is not the consequence of a simple mechanical exclusion.

Table I. Effect of Membrane-rich Fractions and Cell Walls Obtained from Primary Bean Leaves on Tumor Induction by A. tumefaciens strain B6

Samples inoculated	Leaves	Tumors per leaf	Control
	No.	*Mean no.*	%
Experiment 1			
B6 (4.9×10^8/ml)	16	30.1	100
B6 + 13,000*g* ppt (27 mg/ml)[a]	14	33.1	110
B6 + 80,000*g* ppt (14 mg/ml)[a]	16	26.2	87
Experiment 2			
B6 (2.4×10^8/ml)	16	15.9	100
B6 + 13,000*g* ppt (27 mg/ml)[a]	16	14.4	91
B6 + 80,000*g* ppt (14 mg/ml)[a]	14	16.4	103
B6 + cell walls (10 mg/ml)[b]	16	9.3	58

[a]Concentration of membrane fraction as dry weight. Both precipitates represent the amount of material obtained from 2.5 g fresh weight of leaves or about 25 leaves. The 80,000*g* precipitate was obtained after first removing the 13,000*g* precipitate.
[b]Amount of cell wall obtained from 0.1 g fresh weight of leaf.

Table II. Effect of Cell Walls Isolated from Bean Leaves and Their Time of Application on Tumor Initiation by A. tumefaciens strain B6

Materials inoculated[a]		Leaves	Tumors per leaf	Control
First addition	Second addition			
		No.	*Mean no.*	%
B6	None	16	14.3	100
B6 + CW	None	16	3.4	24
B6	CW	16	15.8	111
CW	B6	16	1.8	12

[a]Concentrations applied: B6, about 9×10^8 viable cells/ml; cell walls, 10 mg/ml. Second additions were made about 15 min after the first.

The inhibitory cell wall preparations were treated with heat-inactivated cells of avirulent *A. tumefaciens* strain IIBNV6 which were previously shown to have site attachment capacity by their ability to compete with viable virulent cells for tumor initiation sites (6). After removing most of the IIBNV6 cells from the plant cell wall preparations by centrifugation and washing, the treated plant cell walls were tested for inhibition of tumor formation when mixed and inoculated with strain B6. As shown in Figure 2, the inhibitory activity of these plant walls can be largely eliminated by pretreatment with IIBNV6. Cell walls treated with strain IIBNV6 at about 5×10^9 bacteria/ml was maximal in neutralizing the inhibitory effect of the cell walls. This concentration of bacteria was used in subsequent experiments as a test for inhibition specificity. The somewhat greater inhibition obtained with cell walls treated with 10^9 IIBNV6/ml and the failure to eliminate completely the inhibitory activity of plant cell walls by treatment with IIBNV6 at lower concentrations are probably due to the inability to remove all of the IIBNV6 cells which were not directly attached to the plant cell walls. IIBNV6 cells by themselves are inhibitory (6) because of their ability to compete for wound sites with virulent bacteria.

The specificity of these plant cell wall preparations in prevent-

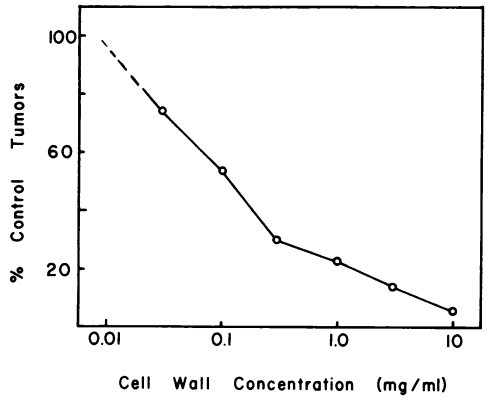

FIG. 1. Titration of the inhibitory activity of bean leaf cell walls on tumor initiation by *A. tumefaciens* strain B6. The concentration of strain B6 in each sample inoculated was 2.2×10^8 viable cells/ml. One hundred per cent control tumors equals a mean of 18.7 tumors/leaf.

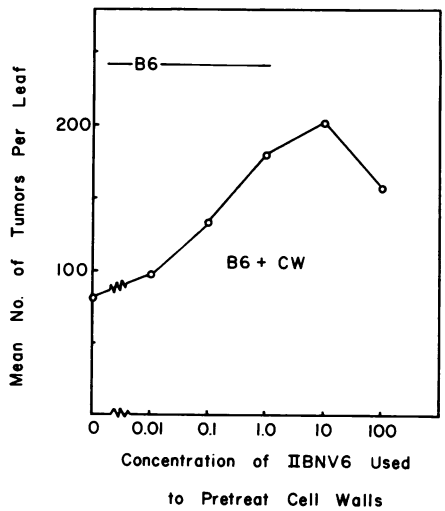

FIG. 2. Effect of treating bean leaf cell walls with different concentrations of heat-killed strain IIBNV6 prior to testing the cell walls for inhibition of tumor initiation by strain B6. All samples contained 1.1×10^9 viable strain B6 cells and samples with cell walls contained the equivalent of 10 mg/ml dry weight of these preparations. IIBNV6 concentration of 1 = 4.8×10^8 cells/ml.

ing tumor induction was further examined as shown in Table III by determining the effects of prior treatment with different bacteria and with LPS from these bacteria. Strains ATCC 6467 and S1005 of *A. radiobacter* were previously shown to lack site-binding activity (6, 13). Treatment of bean cell walls with these bacteria had no effect on the inhibitory activity of the bean leaf cell wall preparations. LPS isolated from strain B6 effectively neutralized the inhibitory effect of the plant cell walls.

The rate of tumor appearance on leaves inoculated with strain B6 or a mixture of strain B6 with plant cell walls was followed to determine if the cell walls only delayed tumor initiation or affected only tumors arising from large or small wounds (8). Table IV shows that the appearance of new tumors was complete by day 8 in both B6 and B6 + cell wall inoculated leaves, as is typical of the response to strain B6 (8). Both early and late appearing tumors were inhibited by the cell walls, although the cell walls had a somewhat greater inhibitory effect on early appearing tumors.

DISCUSSION

The two most probable locations of the host component of *A. tumefaciens* infection site attachment are a portion of the plant cell wall exposed by wounding or an exposed portion of the cell membrane (9). The above data clearly show that mixtures of strain B6 with plant cell wall preparations induce many fewer tumors than control bacteria, whereas comparable amounts of plant membrane fractions had essentially no effect on tumor initiation. The cell wall preparations are inhibitory at relatively low concentration (>0.1 mg/ml) and are effective only when they precede or accompany the infectious bacteria. This inhibitory activity is thus restricted to the period of site attachment, consistent with the cell wall preparations being effective in competing with plant wound sites for virulent bacteria.

The inhibitory action of the plant cell wall cannot be ascribed to simple mechanical blockage of infection sites as pretreatment of these cell wall preparations with avirulent site-binding bacteria or with LPS from virulent bacteria largely eliminates the inhibitory activity of the plant wall preparations. Cells and LPS obtained from non-site-binding agrobacteria had no effect on this activity. Thus, the plant cell wall preparations exhibit a specificity in their ability to inhibit tumor initiation comparable to that characteristic of *in vivo* attachment as determined by direct competition experiments.

The amount of cell wall obtained from a single bean leaf of the size used in the tumor bioassays was about 10 mg dry weight. This concentration of cell wall/ml typically gives about 70% inhibition of tumor initiation and, because 0.1 ml of inoculum was applied per leaf, the amount of cell wall from 0.1 leaf applied to a whole leaf provided this degree of inhibition. Since about 30% inhibition was obtained with cell walls from 0.0005 leaves per inoculated leaf, the range of concentrations over which they inhibit is consistent with their being the natural attachment site.

A somewhat greater portion of early as opposed to late appearing tumors is inhibited by these plant cell wall preparations. Since the over-all period of tumor appearance is similar, it appears unlikely that the proportional change is due to a delay of tumor appearance. The time of tumor appearance depends on initial wound size, the larger wounds giving rise to early appearing tumors (8). Thus, events at larger wounds appear somewhat more susceptible to blockage than those at small wounds. The cause of this anomally is not apparent although it does not detract from the basic conclusion to be derived from this type experiment.

We conclude from these data that wound-exposed portions of host plant cell walls constitute the natural attachment site essential to *Agrobacterium* tumor induction. The apparent attachment of *Agrobacterium* to isolated cell walls which occurs when mixtures of bacteria and cell walls are inoculated reduces tumor initiation, suggesting that attachment *per se* is not sufficient for tumor initiation. To be active in tumor initiation, the site must be localized on a susceptible host cell. The exact nature of the host cell wall components involved in attachment and the subsequent role of this attachment remains to be determined.

Table III. Effect of Pretreating Leaf Cell Wall Preparations with Dead Bacteria or Bacterial Lipopolysaccharide (LPS) on their Ability to Inhibit Tumor Induction

Sample inoculated[a]	Treatment of cell walls	Leaves	Tumors per leaf	Control
		No.	*Mean no.*	%
Experiment 1				
B6	---	16	144.0	100
B6 + CW	None	16	33.8	23
B6 + CW	IIBNV6 (1.6×10^{10})[b]	16	68.0	47
B6 + CW	ATCC 6467 (5×10^{9})[b]	16	21.6	15
B6 + CW	S1005 (8×10^{9})[b]	16	32.9	23
Experiment 2				
B6	---	16	95.5	100
B6 + CW	None	14	27.3	29
B6 + CW	B6 LPS (40 ug/ml)	16	75.9	80
B6 + CW	ATCC 6467 LPS (40 ug/ml)	16	21.9	23

[a]Concentrations of strain B6: Experiment 1, 1.5×10^{9}/ml; Experiment 2, 1.4×10^{9}/ml. Bean leaf cell walls were tested at 10 mg/ml in both experiments.
[b]Cells killed by a 20 min treatment at 60 C, concentrations determined by dilution and plating prior to the heat treatment.

Table IV. Rate of Tumor Appearance on Leaves Inoculated with A. tumefaciens strain B6 in the Presence or Absence of Plant Cell Walls

Time after inoculation	Tumors per leaf		Tumor no. per day
	B6[a]	B6 + cell walls[a]	
Days	*Mean no.*		*Difference*
3	6.5	1.8	- 4.7
4	16.2	3.8	- 7.7
5	29.3	8.7	- 8.2
6	39.0	14.6	- 3.8
7	47.1	19.0	- 3.7
8	49.8	24.5	+ 2.8
9	48.9	25.0	+ 0.5
10	49.8	25.0	---

[a]Concentrations: strain B6, 2.6×10^{9}/ml; cell walls, 10 mg/ml.

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