virulent and avirulent sets of rabies viruses of identical genetic background separated by a minimal number of replication generations. Such sets of viruses may allow a more fruitful search for virus markers associated with virulence or attenuation than have the previously used systems of attenuated viruses compared to virulent ancestors from which they were derived usually by prolonged passage in "unnatural" host systems.

Finally, the mechanism of death induced by rabies virus is incompletely understood, although rabies virus replication is known to occur preferentially in neurons within the central nervous system (*23*). Since neuroblastoma cell lines exhibit various neuronlike physiological and biochemical characteristics, a study of the effect of rabies virus infection on the expression of these neuronal characters in vitro may shed light on the mechanism of rabies virus pathogenesis occurring at the cellular level in vivo.

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Cell Walls of Crown-Gall Tumors and Embryonic Plant Tissues Lack *Agrobacterium* Adherence Sites

Abstract. *Crown-gall tumor initiation by* Agrobacterium tumefaciens *is inhibited by cell walls from normal dicotyledonous plants but not by cell walls from crown-gall tumors apparently because of bacterial adherence or nonadherence, respectively, to the different cell walls. Cell walls from normal and tumor tissues in culture also show this difference, indicating that the two types of tissue stably maintain this difference under these conditions. Habituated tissue cultures, which resemble crown-gall tumor cultures, however, form cell walls that are inhibitory like those of the normal cultures from which they are derived. Monocotyledonous plants do not act as hosts for* Agrobacterium *and bacteria-specific inhibition is not shown by cell walls from several species of grass, a monocot family. Cell walls from "embryonic" tissues (dicot seedlings less than 2 centimeters long), unlike those from older seedlings, are noninhibitory. Crown-gall tumors thus resemble embryonic tissues in this respect.*

The transformation of plant cells to a tumorous state by *Agrobacterium tumefaciens* proceeds only after attachment of the bacterium to a site in a plant wound (*1*). Isolated cell walls from susceptible pinto bean leaves were found to exhibit the characteristics expected of the host adherence site (*2*). When these cell walls were inoculated with virulent bacteria on bean leaves, they reduced the number of tumors initiated, apparently by their ability to bind bacteria and thus reduce the number of free bacteria available to attach to actual wound sites where tumor initiation could occur. These cell walls were noninhibitory if added 15 minutes after the bacteria, when bacterial adherence is complete (*1*), or if they were pretreated with an avirulent site-binding strain of bacteria. In examining the effect of cell walls isolated from different plant species, plant parts, and plant tumors, remarkable differences were found in the ability of plant cell walls to inhibit tumor initiation.

Using the procedure of Nevins *et al.* (*3*), we isolated cell walls from 7- to 21-day-old seedlings, "embryonic" tissues from seeds germinated for 3 days (total length < 2 cm), crown-gall tumors in vivo, normal tissues from the same plants, and normal, habituated, and crown-gall tumor tissue cultures. The tissues were blended with an equal volume of 0.5*M* potassium phosphate buffer, *p*H 7.0, and the cell walls were collected by centrifugation for 15 minutes at 2000*g*. They were washed by resuspension and centrifugation three times in this buffer, homogenized with acetone, collected by filtration, and washed three times with fresh acetone. Remaining acetone was removed by overnight evaporation and the dry cell walls were stored in a desiccator.

For infectivity tests, dry cell walls were resuspended in 0.05*M* potassium phosphate buffer, *p*H 7.0, and mixed with an equal volume of washed *A. tumefaciens* strain B6 suspended in the same buffer (*2*). The concentration of cell walls in the samples inoculated was 10 mg (dry weight) per milliliter and the concentration of B6 was 10^8 to 5×10^8 viable bacteria per milliliter. To show that inhibitory effects of cell walls were specific for *Agrobacterium*, a portion of each cell wall preparation was treated with avirulent site-binding strain IIBNV6 of *A. tumefaciens* (5×10^9 cells per milliliter) for 15 minutes. The plant cell walls were then collected by a 10-minute centrifugation at 2000*g*, sus-

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pended in phosphate buffer, and centrifuged again, and the precipitated cell walls were resuspended in their original volume and mixed 1:1 (by volume) with strain B6 for inoculation. The nonpretreated cell walls received similar treatment but without IIBNV6. This washing per se had no discernible effect on the activity of the plant cell walls in the infectivity assay.

Samples composed of strain B6 plus cell walls, B6 plus IIBNV6-pretreated cell walls, and B6 alone as a control were each inoculated on 14 to 16 primary leaves of 7-day-old pinto bean plants by a Carborundum wounding procedure (*4*). Seven days later the tumors were counted. A linear relation between bacterial concentration and mean tumor number per leaf is obtained in this bioassay (*4*), with standard errors which are typically less than ± 20 percent of the mean.

Table 1 lists the different sources of cell walls tested in the tumor bioassay according to their ability to inhibit tumor initiation and to be partly neutralized by IIBNV6 pretreatment, or to be noninhibitory and show the opposite response after IIBNV6 pretreatment. Data from two illustrative experiments are shown in Table 2. Cell walls from normal and habituated tissue cultures of sunflower and from 7-day-old primary pinto bean leaves were inhibitory, whereas those from three parts of very young bean seedlings (embryonic tissues) and a B6 sunflower tumor tissue culture were noninhibitory. Pretreatment of the inhibitory cell walls with strain IIBNV6 greatly reduced their inhibitory effect, but similar treatment of the noninhibitory cell walls typically made them somewhat inhibitory, probably because of incomplete removal of IIBNV6, which can directly compete with B6 for host sites (*1*).

Figure 1 shows the means of the determinations for the different types of cell walls listed in Table 1. Normal dicot tissues, whether from 7-day or older plants or from tissue cultures, always yielded cell walls which inhibited tumor initiation. In pinto beans all parts tested, roots, hypocotyls, epicotyls, and primary leaves, were equally inhibitory and, as with other dicots, this activity was largely neutralized by the IIBNV6 pretreatment. This was also true of the cell walls from habituated tissue cultures which, although derived from normal hormone-requiring cultures, have lost the hormone requirements for growth in vitro and in many respects behave like crown-gall tumor tissue cultures (*5*).

To be inhibitory these cell walls must act either on the bacterium or on the prospective tumor cells. The cell walls have no effect on tumor initiation when added 15 minutes after strain B6, when bacterial site attachment is complete (*2*), and their inhibitory activity can be neutralized by pretreatment with either IIBNV6 or lipopolysaccharide, the bacterial site-binding component isolated from this and other-site-binding *Agrobacterium* strains (*6*). These results are consistent, therefore, in indicating that inhibition arises as a result of strain B6 adherence to surfaces of the cell wall fragments rather than from direct effects on the plant host cells.

Table 1. Source and type of response of plant cell walls tested for inhibition of tumor initiation by *Agrobacterium tumefaciens* strain B6.

Inhibitory cell walls	Noninhibitory cell walls
Normal plant tissue cultures	Crown-gall tumor tissue cultures*
Havana tobacco, stem	Havana tobacco—B6
Xanthi tobacco, stem	Wisconsin 38 tobacco—B6
Vinca, stem	Xanthi tobacco—B6806 (two isolates)
Pinto bean, leaf (two isolates)	Xanthi tobacco—A208
Sunflower, stem	Vinca tumors—IIBV7, A6, B6, T37, W1
Carrot, root	Pinto bean tumors—181, H100, B6
Potato, tuber	Sunflower—B6
Normal dicotyledonous plants (in vivo)	Crown-gall tumors (in vivo)*
Pinto bean, leaves, hypocotyls, roots, epicotyls	Tomato, stem—B6
Peas, leaves plus hypocotyl	Carrot, root—B6
Kalanchoe, leaves	Tobacco, stem—B6
Carrot, root	Kalanchoe, leaf—B6
Castor bean, cotyledonary leaves	"Embryonic" dicotyledonous tissues
Tomato, shoot and stem	Pinto bean, root
Tobacco, stem	Pinto bean, leaf plus epicotyl
Habituated tissue cultures	Pinto bean, hypocotyl
Pinto bean, leaf	Pea, entire embryo
Wisconsin 38 tobacco, stem (two isolates)	Castor bean, entire embryo
Sunflower, stem	Normal monocotyledonous plants (in vivo)
Carrot, root (two isolates)	Barley, shoots (four varieties)
Havana tobacco, stem (three isolates)	Corn, shoots
	Oat, shoots
	Wheat, shoots

**Agrobacterium* strains used to induce the different tumor lines are indicated after dashes.

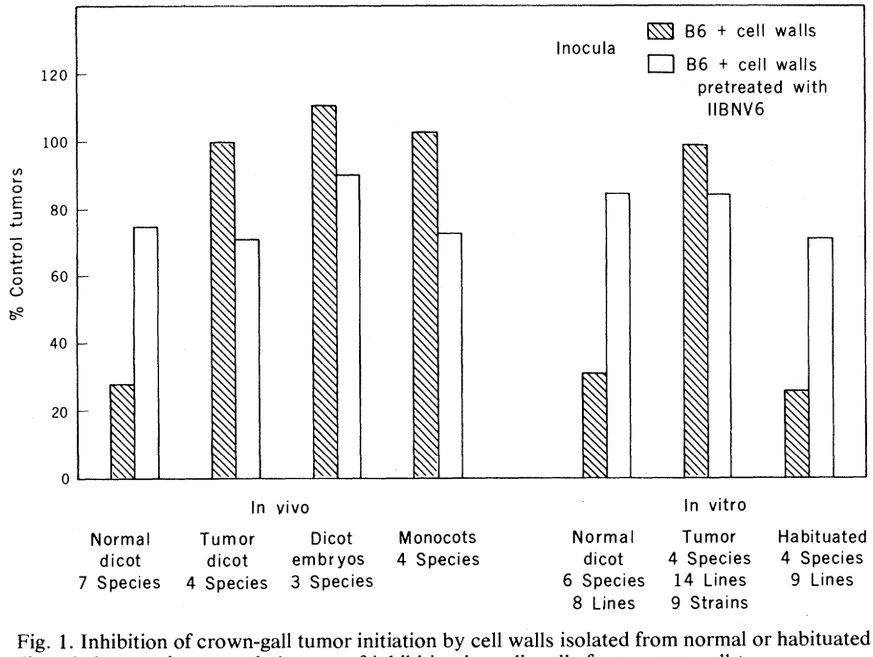

Fig. 1. Inhibition of crown-gall tumor initiation by cell walls isolated from normal or habituated dicotyledonous tissues and absence of inhibition by cell walls from crown-gall tumors, monocotyledonous tissues, and embryonic dicotyledonous tissues. Mean values of the determinations obtained with each class of cell walls are shown. Standard errors for the bars were less than ± 7 percent, except for those shown for monocots, which were ± 22 percent (B6 plus cell walls) and ± 20 percent (B6 plus cell walls pretreated with IIBNV6), due largely to the three inhibitory barley cultivars.

Cell walls from crown-gall tumors in vivo or from tissue cultures established from such tumors, however, were noninhibitory (Fig. 1). These represent tumors induced by nine strains of *Agrobacterium* on eight species of plants (Table 1). Cell walls from very young seedlings (< 2 cm long) of newly germinated pinto beans, peas, and castor beans were also noninhibitory, although in each case those from comparable 7- to 10-day-old seedlings were inhibitory. Cell walls from four species of monocots responded like these tumor and embryonic cell walls, although those from three of the four varieties of barley showed some inhibition. However, tenfold higher concentrations of barley cell walls were necessary to equal the effect obtained with cell walls from pinto beans, and barley cell walls treated with IIBNV6 were even more inhibitory, which suggests that their inherent inhibitory effect was due to something other than their ability to bind *Agrobacterium*.

Table 2. Effect of cell walls (CW) from several plant tissues on tumor initiation when mixed and inoculated with *A. tumefaciens*. Each value in the second column is the mean ± standard error (N = 16).

Source of cell walls inoculated with strain B6	Tumors per leaf	Percentage of control
Experiment 1		
None	101.1 ± 11.3	100
Pinto bean leaf (7 days old) CW	26.5 ± 4.1	26
Same pretreated with IIBNV6	70.4 ± 7.9	70
Pinto bean "embryo" root CW	115.7 ± 10.2	114
Same pretreated with IIBNV6	75.4 ± 7.3	75
Pinto bean "embryo" leaf plus epicotyl CW	113.7 ± 12.3	112
Same pretreated with IIBNV6	92.8 ± 10.9	92
Pinto bean "embryo" hypocotyl CW	120.1 ± 14.7	119
Same pretreated with IIBNV6	88.8 ± 10.1	88
Experiment 2		
None	65.5 ± 13.3	100
Normal sunflower tissue culture CW	26.4 ± 3.5	40
Same pretreated with IIBNV6	57.1 ± 6.8	87
Habituated sunflower tissue culture CW	22.8 ± 3.8	35
Same pretreated with IIBNV6	63.8 ± 8.2	97
Tumor (B6) sunflower tissue culture CW	57.4 ± 8.0	88
Same pretreated with IIBNV6	44.2 ± 6.0	67

These data demonstrate that cell walls isolated from crown-gall tumors differ from those obtained from normal tissues in a way which affects the ability of *Agrobacterium* to adhere to the walls. This difference exists in vivo and is maintained when the tissues are grown in culture. Habituated tissues, which resemble crown-gall tumor tissues in their growth requirements in vitro and in some cases in their ability to proliferate as unorganized tissues when grafted to normal plants (*5, 7*), nevertheless retain the cell wall characteristics of normal tissues. This effect of cell walls in these infectivity tests thus provides a new means of ascertaining whether a tissue culture is of crown-gall or normal origin.

Because the cell walls from very young dicot seedlings were noninhibitory and those from seedlings only 4 days older were inhibitory, a developmental change in cell wall metabolism must occur in this interval in the root, hypocotyl, and shoot. This suggests that there is a cell wall metabolism peculiar to embryos and seedlings during the first days of germination which changes to a mature type of cell wall metabolism. The lack of inhibitory activity on the part of both embryonic and tumor cell walls may be due to a similarity in their cell wall metabolism, and therefore an embryonic-type cell wall metabolism may characterize the tumors. Pectinesterase treatment of these cell walls suggests that this similarity exists at the biochemical level since both types of cell walls become inhibitory after enzyme treatment (*8*). Analogous relations between embryonic and cancer cells are found in animals (*9*) and suggest yet another parallel between crown-gall and animal cancers.

Cell walls from the four species of monocots, all belonging to the grass family (Table 1), also did not show IIBNV6-reversible inhibition. These grasses and monocots in general are resistant to *Agrobacterium* infection (*10*). Because site attachment is essential for tumor initiation (*1*), the apparent absence of adherence sites in these monocot cell walls suggests that failure of the bacterium to complete the attachment step may be an effective barrier to *Agrobacterium*-induced tumor formation in these plants. Other barriers may also exist, however, and additional monocots need to be examined to determine whether this difference is representative for all monocots. After pectinesterase treatment these monocot cell walls become inhibitory (*8*), as do the cell walls of tumor and embryonic dicot tissues described above. The similar responses obtained with the monocot and embryonic dicot cell walls raise the interesting speculation that in association with the evolutionary separation of these two groups, an embryonic type of cell wall metabolism may have become characteristic of the entire monocot life cycle.

The adherence of agrobacteria to host cell walls depends on the polysaccharide portion of lipopolysaccharide molecules on the outer surface of the bacterium (*11*) interacting with the outer pectin-rich layer of the host cell walls (*2, 8*). Resolution of the nature of the components of this pectic layer involved in adherence and demonstration of the actual chemical or structural changes in the biosynthesis of these components in tumor induction, in seedling germination, and in monocots will be important in establishing many of the relationships suggested by these data. This may also provide a much needed biochemical test which is definitive for all crown-gall tumors.

Note added in proof: Data on cell walls from six monocots belonging to four additional families were obtained while this report was in press. In each case the response obtained before and after IIBNV6 pretreatment was similar to those reported for the grasses. The sources of these cell walls were asparagus, shoot; Spanish onion, bulb; green onion, leaf; philodendron, leaf; banana palm, leaf; and *Aechmea* sp., leaf.

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Switch in Immunoglobulin Class Production Observed in Single Clones of Committed Lymphocytes

Abstract. *Mouse spleen cells, after stimulation with lipopolysaccharide, were cloned in culture. After 4 to 5 days, the daughter cells were stained and examined for immunoglobulin class with double immunofluorescent reagents. A switch of the stained color of these cells was observed, implying a switch from imunoglobulin M to immunoglobulin G production in the progeny of a single B cell.*

After an antigen is injected into a mouse, the first antibodies to appear in the serum are of the immunoglobulin M (IgM) class; these are followed by immunoglobulin G (IgG). This so-called switch in immunoglobulin (Ig) class expression occurs also in cultures of mitogen-stimulated B cells (bone marrow–derived lymphocytes) (*1*). Spleen cell cultures stimulated by bacterial lipopolysaccharide (LPS) contain mostly IgM-producing cells at the beginning, but a few hours after stimulation cells that produce both IgM and IgG (double producers) appear. The number of double producers increases with time; later, however, cells producing only IgG appear (*2*).

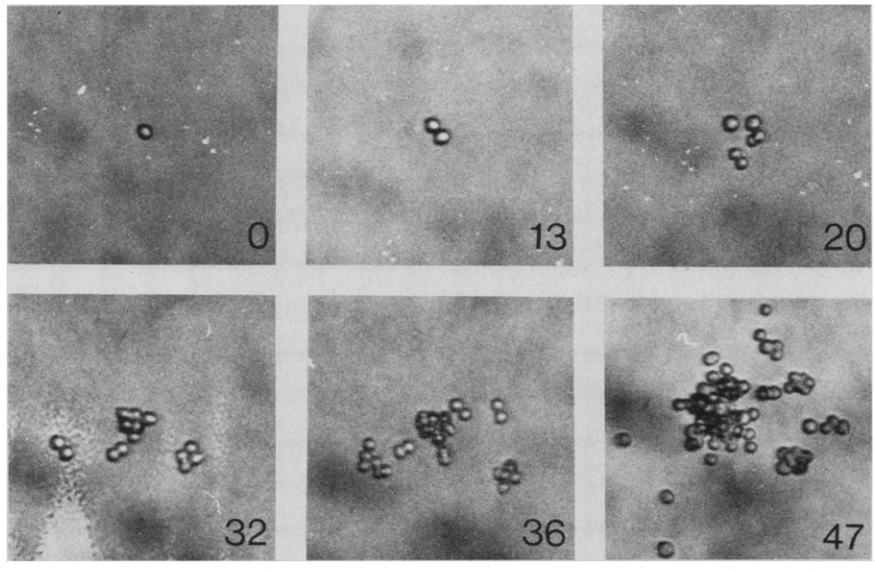
Since the presence of antiserums against the μ chain (the chain characteristic of IgM) throughout the culture period suppresses the appearance of IgG without affecting growth stimulation (*3*), it has been suggested that IgG-producing cells must derive from IgM-producing precursors. However, alternative explanations involving cooperation between cells carrying μ chains and precursors of cells carrying γ chains have not been formally excluded. We now present evidence based on single-cell experiments indicating that cells producing only IgM can give rise to daughters producing IgG.

Mouse spleen cells (2.5×10^5 cell/ml; BALB/c or C57 strain) were stimulated with LPS (20 μg/ml) in mass culture (*1*). When blast formation began, at 1 or 2 days, single blasts were withdrawn by micromanipulation and placed in Terasaki wells over a feeder layer of LPS-stimulated spleen cells in 0.3 percent agar.

Without a feeder layer, the cells divided three times at most. With a feeder layer, between 1 cell and 200 cells per well were produced 4 or 5 days after mitogenic stimulation of the single cell (Fig. 1). The shortest generation time observed was 8 to 9 hours (Figs. 1 and 2). The classes of Ig produced by 19 of the larger clones were analyzed by double immunofluorescence to detect μ- and γ-specific determinants, by a method that

Table 1. Double immunofluorescence staining for μ and γ chains. Of the 19 clones studied, five were heterogeneous. The cells were fixed for 5 minutes in ethanol before they were stained. Therefore both cytoplasmic and membrane immunoglobulins were detected. On day 4 or 5 the cells showed mainly cytoplasmic immunoglobulin. For clones 1 to 3, staining for IgG_{2a} and IgG_{2b} was not differentiated; for clones 4 and 5, only staining for IgG_{2a}, and not for IgG_{2b}, was done.

Clone	Number of cells				Remarks
	$\mu-\gamma-$	$\mu+\gamma-$	$\mu+\gamma+$	$\mu-\gamma+$	
1	5	31	3	15	IgG_2, no IgG_1
2	0	22	7	0	IgG_1, no IgG_2
3	0	28	1	0	IgG_1, no IgG_2
4	3	40	1	11	IgG_{2a}
5	1	15	8	5	IgG_{2a}



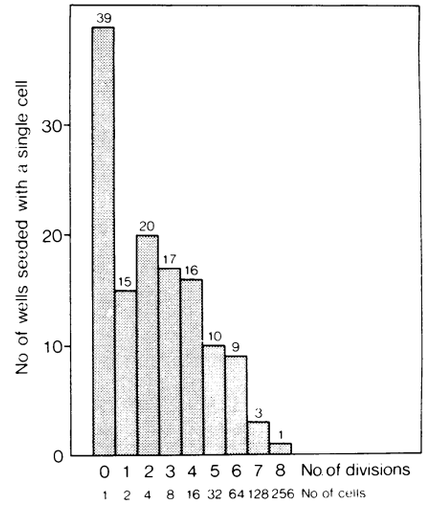


Fig. 1 (left). Clonal expansion of a single cell in culture. At day 2, after mitogenic stimulation, a blast cell was placed in a Terasaki well over a 2-day-old LPS-stimulated feeder culture. Numbers in the right lower corner represent the hours after seeding. Fig. 2 (right). Distribution of clone size. Clonal expansion of 130 single cells in culture. The cell numbers in a clone were rounded to the nearest power of 2.

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