Involvement of Carrot Cell Surface Proteins in Attachment of Agrobacterium tumefaciens

Received for publication October 17, 1986

ROBIN H. G. GURLITZ, PATRICIA W. LAMB, AND ANN G. MATTHYSSE*
Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27514

ABSTRACT

The initial step in tumor formation by Agrobacterium tumefaciens is the site-specific attachment of the bacteria to plant cells. A similar attachment to plant tissue culture cells has been observed. Binding to carrot suspension culture cells was not dependent on the presence of divalent cations and was not inhibited by the addition of mannose, α-methyl mannoside, galactose, arabinose, glucosamine, 2-deoxyglucose, or 0.25 molar NaCl to the culture medium. The ability of the carrot cells to bind A. tumefaciens was markedly reduced by elution of the cells with dilute detergent or CaCl₂ or by incubation of the cells with proteolytic enzymes. The carrot cells were not killed by these treatments and recovered the ability to bind A. tumefaciens within 3 to 6 hours. A. tumefaciens did not bind to carrot cells which had been induced to form embryos (AG Matthysse, RHG Gurlitz 1982 Physiol Plant Pathol 21: 381-387). A comparison of the peptides eluted from embryos and from uninduced cells using sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that there were several changes in extractable polypeptides after embryo induction. One or more of the polypeptides present before embryo induction and absent from embryos may be involved in the binding of A. tumefaciens to the carrot cell surface.

One of the initial steps in tumor formation by Agrobacterium tumefaciens is the site-specific attachment of the bacteria to plant host cells (4). A similar, specific attachment of A. tumefaciens to plant tissue culture cells has been demonstrated in several laboratories (2, 10, 15). This article reports studies on the receptor on the surface of carrot suspension culture cells to which the bacteria bind. Previous research has shown that A. tumefaciens attacked both to living and killed carrot cells (8) and to carrot protoplasts (9). The bacteria did not bind to carrot embryos 24 h after their induction from the suspension culture cells (7). These observations suggest that the carrot cell receptor for attachment of A. tumefaciens is present on the carrot cells before the addition of the bacteria to the culture rather than induced by the presence of the bacteria and that the carrot cells do not play an active role in the attachment process. The fact that A. tumefaciens no longer bound to suspension culture cells 24 h after the induction of these cells to form embryos suggests that the receptor either was degraded, modified, or became inaccessible during embryo formation (7).

The nature of the receptor on the host cell surface to which A. tumefaciens binds is unknown. Ohyama et al. (15) found that the binding of A. tumefaciens to Datura cells was not dependent on divalent cations. Although tumor formation on bean leaves (5) and potato discs (17) has been reported to be inhibited by polygalacturonic acid and pectin, no direct inhibition of attachment to tissue culture cells by these compounds has been observed (9).

Attachment of wild-type strains of A. tumefaciens to the surface of carrot cells is accompanied by bacterial synthesis of cellulose fibrils and the formation of large aggregates of bacteria on the plant cell surface (8). Only a few of the bacteria in these aggregates are attached directly to the carrot cell surface. The remainder of the bacteria are held to the carrot cell indirectly by the bacterial cellulose fibrils. Thus, it is not possible to estimate the number of receptor sites for A. tumefaciens on a carrot cell by measuring the attachment of wild-type bacteria. The isolation of a mutant of A. tumefaciens strain A6, which does not make detectable amounts of cellulose, allows the measurement of the number of bacteria directly bound to receptors on the surface of the carrot cell (6). We report below the results of these measurements which can be used to estimate the average number of receptors for A. tumefaciens per carrot suspension culture cell. In addition, we have examined the possible role of divalent cations, carbohydrates, and proteins in the binding of the bacteria to carrot cells.

MATERIALS AND METHODS

Sources and Growth of Cultures. Bacteria were grown and viable cell counts were determined as described previously (10). Virulent Agrobacterium tumefaciens A6, an octopine strain, and C58, a nopaline strain, were obtained from Dr. Armin Braun, Rockefeller University and Dr. Mary-Dell Chilton, Washington University, respectively. The bacterial mutant strain Ce-12, which does not synthesize any detectable cellulose, was obtained by transposon mutagenesis of strain A6 (6). Normal and embryogenic suspension cultures of carrot, Dacus carota, were provided by Dr. Wendy Boss, North Carolina State University. Normal carrot was grown in Murashige and Skoog medium (13) with weekly transfers as described previously (8). Embryogenic carrot was grown in wild carrot medium (20). Carrot embryo formation was induced as described previously (7, 20).

Measurements of Bacterial Attachment. Bacterial attachment to carrot cells was measured after transfer of the cells into fresh Murashige and Skoog medium (13) unless otherwise specified. Sugars were filter sterilized and added to the medium. To determine whether there was any effect of divalent cations on attachment, measurements were made in Murashige and Skoog medium with 1 mM EDTA or 1.3 mM EGTA (pH 5.9) to remove divalent cations, collected by filtration, and resuspended in Murashige and Skoog medium without Ca²⁺ and Mg²⁺. The alterations in the medium had no effect on the viability of the bacteria or of the carrot cells except the addition of 0.25 mM NaCl, which caused...
plasmolysis of the carrot cells but had no effect on bacterial viability. In general, the bacteria had a 2 to 3 h lag time before they began to grow when they were transferred from Luria broth in which they were grown into plant tissue culture media (10). Thus, the number of viable bacteria changed <5% during the 60 min incubation in the various media with carrot cells. For kinetic experiments bacteria were added to the carrot cell suspension to a final concentration of 1 to 4 x 10^6 per ml unless otherwise specified. Bacteria were incubated with the carrot cells for varying times, and the free bacteria were separated from the carrot cells and bound bacteria by filtration through a Miracloth filter. Numbers of free and attached bacteria were determined by viable cell counts (10). Kinetic measurements were made during the first 2 h of incubation of the bacteria with the plant cells, because the bacterial synthesis of cellulose fibrils induced by the plant can result in the nonspecific adherence of the bacteria to surfaces such as filter paper after prolonged incubation times (8). For microscopic observations, bacteria were added to a final concentration of 2 to 10 x 10^6 per ml. Carrot cells were present at a concentration of 1 to 3 x 10^6 cells per ml. For photomicroscopy a Zeiss photoscope 2 and Nomarski optics were used to photograph living cells. Attachment measurements were based on both microscopic observations and on kinetic measurements of plasmolysis.

Estimation of the Number of Receptor Sites for A. tumefaciens per Carrot Cell. To estimate the number of bacteria bound directly to the carrot cell surface, the binding of a cellulose-minus mutant of A. tumefaciens, Ce-12, which is capable only of direct binding to the plant cell, was determined at varying ratios of bacteria to carrot cells. The number of bacteria inoculated ranged from 10^3 to 10^5 per ml and the number of carrot cells from 10^4 to 10^6 per ml.

Treatments of the Carrot Cells. For some attachment studies the carrot cells were digested with filter-sterilized 0.1% trypsin (Sigma), 0.1% chymotrypsin (Sigma), or 0.01% proteinase K (EM Biochemicals) in Murashige and Skoog medium (pH 7.5) for 1 h prior to the addition of the bacteria. The carrot cells were collected by filtration, rinsed three times with 10 volumes of Murashige and Skoog medium (pH 5.7), resuspended in fresh medium at a concentration of 1 to 2 x 10^6 cells per ml, and used for attachment studies, either immediately or after shaking. Microscopic observations showed that the cells were intact and had normal cytoplasmic streaming after these treatments. Carrot cells which had been treated with trypsin were resuspended in Murashige and Skoog medium containing 10 μg soybean trypsin inhibitor/ml. The addition of soybean trypsin inhibitor to the medium with untreated carrot cells had no effect on the rate of bacterial attachment.

For some studies, surface components of carrot suspension culture cells were removed by eluting the cells with 0.01 to 0.1% Triton X-100 for 45 to 60 min or with 0.1 to 0.2 M CaCl_2 for 1 h. The carrot cells were collected by filtration after these treatments, washed with Murashige and Skoog medium, resuspended in fresh medium at a concentration of 1 to 2 x 10^6 cells per ml, and used for attachment studies either immediately or after shaking. Microscopic observations showed that the carrot cells were intact and showed normal cytoplasmic streaming after extraction with Triton X-100. Carrot cells were plasmolysed after elution with 0.2 M CaCl_2 but the cells recovered rapidly when resuspended in Murashige and Skoog medium.

Analysis of Surface Components Eluted from Carrot Cells. The filtrates from the elution of carrot cells with 0.1% Triton X-100 or 0.1 M CaCl_2 were dialyzed against 10 mM Tris-HCl (pH 7.0) and concentrated 50- to 100-fold with aqueacide I-A (Calbiochem). Concentrated eluates were analyzed by SDS-PAGE by the method of Laemmli (3). The gels were stained with silver by the method of Merril et al. (12). When the eluates were treated with 100 μg proteinase K/ml for 60 min prior to loading the gel, only bands attributable to proteinase K were visible on the gel, suggesting that the silver-stained bands produced by undigested eluates were due to polypeptides.

RESULTS

Effects of Alterations of the Medium on Attachment. Agrobacterium tumefaciens bound to carrot suspension culture cells when they were incubated together in Murashige and Skoog medium. There appeared to be no requirement for divalent cations for bacterial attachment (Table I). Carrot cells washed with 1.3 mM EGTA or 1 mM EDTA and incubated with A. tumefaciens strains A6 or C58 in medium without Ca^{2+} and Mg^{2+} still bound bacteria. Bacteria also attached to carrot cells when the incubation medium contained 1 mM EDTA, but prolonged incubation in the presence of EDTA caused plasmolysis of the carrot cells and a decrease in bacterial viability for both strains A6 and C58.

The addition of a high concentration of salt, 0.25 M NaCl, to the incubation medium also had no effect on bacterial attachment, suggesting that the attachment was not ionic. The addition to the medium of several sugars and sugar derivatives, including mannose and α-methyl mannoside, which inhibit bacterial attachment mediated by type I pili (16, 18), and 2-deoxyglucose, which inhibits attachment of Rhizobium trifolii to clover (1), was also without effect on bacterial attachment (Table I). Results similar to those shown in Table I were obtained with A. tumefaciens strain C58 as well as with strain A6 and were observed with both strains kinetically as well as microscopically with the larger bacterial inoculum (1-2 x 10^9 bacteria per ml) required for microscopic studies.

Location of the Receptor Site. We have reported previously that A. tumefaciens strains A6 and C58 attached both to intact carrot suspension culture cells and to protoplasts derived from these cells (9). To determine whether the receptor site for the bacteria was in fact located on the carrot cell wall or was only exposed in the vicinity of plasmodesmata, or of previous plasmadesmata which were broken when the cells separated, we examined the attachment of bacteria to carrot cells which were plasmolysed by the addition of 0.4 M mannitol to the Murashige and Skoog medium 15 min before the addition of the bacteria. As seen in Figure 1, bacteria were observed attached to the carrot cell wall in regions in which the plasmalemma had withdrawn from the cell wall. In addition, plasmolysis of the plant cells in 0.25 M NaCl or in 0.4 M mannitol had no significant effect on

![Table I. Effect of the Medium Composition on the Attachment of A. tumefaciens Strain A6 to Carrot Cells](data:image/png;base64,Little image content)

*Mean ± SD. Attachment of bacteria in the control medium was between 36 and 50% of the bacteria inoculated. Attachment was measured after 60 min incubation. Each experiment was repeated a minimum of three times. 
* Carrot cells were washed with 1 mM EDTA or 1.3 mM EGTA for 15 min prior to their addition to the medium.
bacterial attachment measured kinetically (Table I). Results similar to those shown with strain A6 were obtained with strain C58. Thus, a receptor for attachment of *A. tumefaciens* appears to be located on the surface of the carrot wall.

**Estimation of the Number of Receptor Sites for *A. tumefaciens* per Carrot Cell.** A virulent transposon mutant of *A. tumefaciens* strain A6, which does not make cellulose, Ce-12, was used to estimate the number of bacteria which could bind directly to the carrot cell surface. When wild type *A. tumefaciens* are incubated with an excess of plant tissue culture cells (1–100 plant cells per bacterium), 35 to 60% of the bacteria inoculated bind to the host cells (2, 8, 10, 15). The actual percentage depends on the strain of *A. tumefaciens* tested. The reasons for the lack of 100% binding of any strain of *A. tumefaciens* are unknown. When the ratio of the number of Ce-12 mutant bacteria to the number of carrot cells in the incubation mixture was varied from 1 bacterium for every 100 carrot cells to $10^3$ bacteria for every carrot cell about 20% of the bacterial inoculum was bound to the carrot cells after incubation for 60 min (Fig. 2). (Binding of strain Ce-12 is complete by this time [6].) At $10^3$ bacteria per carrot cell the binding of 20% of the bacterial inoculum corresponds to 200 bacteria bound per carrot cell. When the ratio of bacteria to carrot cells was increased to $10^4$ bacteria per carrot cell only about 2% of the bacterial inoculum was bound. This suggests that the average carrot suspension culture cell could bind about 200 bacteria.

**The Nature of the Carrot Cell Receptor Sites for the Binding of *A. tumefaciens*.** When carrot suspension culture cells were extracted with 0.01 to 0.1% Triton X-100, 80% of the receptor for binding of *A. tumefaciens* was removed, and binding of the bacteria to the carrot cells was reduced (Table II). The carrot cells appeared to be undamaged by this extraction; their appearance in the light microscope was unaltered and they showed normal cytoplasmic streaming. When the carrot cells were incubated in Murashige and Skoog medium after the Triton extraction, they appeared to recover, and 3 h later they showed normal ability to bind *A. tumefaciens* (Table II). Similar results were obtained by extracting the carrot cells with 0.1 to 0.2 M CaCl$_2$, which has been reported to extract proteins from plant cell wall preparations (19). Treatment of the carrot cells with proteolytic enzymes also reduced their ability to bind *A. tumefaciens* (Table II; Fig. 3). The cells appeared normal after these treatments and recovered their ability to bind *A. tumefaciens* by 6 h after treatment with trypsin or chymotrypsin. When 0.5 µg/100 mg cycloheximide/ml was added to the medium after treatment with trypsin, the carrot cells failed to recover the ability to bind the bacteria and only bound 20 ± 10% of the bacteria bound by the control bacteria. This result suggests that, after trypsin treatment, plant cells require protein synthesis for the recovery of their ability to bind the bacteria.

The proteins eluted from the surface of the carrot cells with Triton X-100 and CaCl$_2$ were examined by SDS-PAGE. About 20 bands were visible in these eluates by a silver staining procedure to visualize the proteins. Proteins were also eluted by an identical procedure from a line of embryogenic carrot cells that binds *A. tumefaciens* and from the same line of embryogenic carrot cells 24 h after they were induced to form embryos. These carrot embryos do not bind *A. tumefaciens* (7). Several changes in the elutable proteins were observed when comparing the normal carrot suspension cells, embryogenic carrot cells, and carrot embryos (Fig. 4). Any of the proteins present in the first two eluates and absent in the third eluate is a candidate for a protein involved in the receptor site for *A. tumefaciens*. However, the bands visible on these gels represent only the more abundant proteins of the carrot cell surface.
The effect of trypsin treatment of intact carrot cells on the polypeptides extractable by Triton X-100 was examined. No polypeptide bands except those attributable to trypsin were seen after SDS-PAGE of Triton extracts of carrot cells which had previously been treated with trypsin (Fig. 5), suggesting that trypsin treatment removes the Triton-extractable polypeptides from the carrot cells.

FIG. 3. Attachment of A. tumefaciens strain A6 to carrot cells. (X), Untreated carrot cells; (O), carrot cells treated with 0.1% trypsin immediately prior to the addition of the bacteria; (•) carrot cells treated with 0.1% chymotrypsin immediately prior to the addition of the bacteria. Bars indicate standard deviation of a minimum of three experiments. Incubation mixtures contained 1 to 3 × 10⁶ carrot cells per ml and 1 to 3 × 10⁶ bacteria per ml.

FIG. 4. SDS-PAGE of proteins extracted from: 1, carrot embryos 24 h after embryo induction of the embryogenic suspension culture; 2, embryogenic carrot suspension culture cells; and 3, carrot suspension culture cells. Each channel contains a 0.1% Triton X-100 extract of 3 to 5 × 10⁶ cells. A 3% stacking and 12% acrylamide running gel were used. The positions of protein standards of known mol wt separated on the same gel are shown at the left. The numbers represent mol wt in kD. Arrows indicate bands seen in extracts of suspension culture and embryogenic suspension culture cells and absent in extracts of carrot embryos.

FIG. 5. SDS-PAGE of proteins extracted from: (a), carrot suspension culture cells, and (b), carrot suspension culture cells treated with 0.1% trypsin. Channel (c) contains 0.5 mg of trypsin. Channel (a) contains an extract from 2 × 10⁶ cells. Channel (b) contains an extract from 2 × 10⁶ cells. A 3% stacking and 11% acrylamide running gel were used. The position of protein standards of known mol wt is shown at the left. The numbers represent mol wt in kD.

DISCUSSION

A receptor to which A. tumefaciens binds is apparently present on the carrot cell wall since the bacteria bound to the walls of plasmolyzed carrot cells in regions in which the plasmalemma no longer made contact with the cell wall (Fig. 1). The question of the identity or nonidentity of the receptor on the plasmalemma of carrot protoplasts with this receptor on the cell wall remains unresolved.

In agreement with the results of Ohyama et al. (15) with Datura cells, bacterial binding to the carrot cell wall was not dependent on divalent cations. The binding was not inhibited by mannose and thus probably did not involve type I pili on the bacterium, since binding of type I pili is generally inhibited by mannose (16, 18). Bacterial binding was also not inhibited by high ionic strength (0.25 M NaCl), which inhibits binding of hydroxyproline-rich glycoproteins from tobacco cells to Pseudomonas solanacearum (11), nor was binding inhibited by 2-deoxyglucose which inhibits binding of trifolin A from clover to R. trifolii (1). Other sugars, such as galactose and arabinose, found in the plant cell wall also failed to inhibit binding of the bacteria. Thus, the binding of A. tumefaciens to carrot cells appears to involve a receptor site different from those previously
characterized for other bacteria.

Some of the receptor activity could be removed from the carrot cell surface by extraction with detergent or CaCl2 or by digestion with proteases. The carrot cells were not killed by these treatments and, after 3 to 6 h of incubation, they recovered the ability to bind A. tumefaciens. This recovery did not occur if cycloheximide was included in the incubation medium. Thus, it appears that a protein (or proteins) may be a part of the receptor site. When a comparison is made of the polypeptides eluted by detergent from carrot suspension culture cells, which bind A. tumefaciens, with the polypeptides eluted from carrot embryos, which do not bind A. tumefaciens, several differences were observed in the bands seen after SDS-PAGE. Any of these bands present in the eluates of suspension culture cells and absent in the eluates of embryos may be polypeptides involved in the binding of A. tumefaciens.

Our results differ from those of Neff and Binns (14) who found that treatment of tomato suspension culture cells with trypsin did not affect their ability to bind A. tumefaciens. However, their measurements of bacterial binding were made 3 h after the treatment. Our results suggest that the tomato cells might have at least partially recovered from the treatment by this time. In addition, the receptor on the surface of tomato cells might be less accessible to the enzyme for a variety of reasons, including the possibility that it could be glycosylated in tomato. Tomato cell wall preparations were found to be inhibitory to the binding of the bacteria. This inhibitory activity was reduced by treatment of the cell wall preparations with trypsin or with pronase (14).

The results of experiments in which the binding of a cellulose-minus mutant strain of A. tumefaciens to carrot cells was measured suggest that only about 200 bacteria can be bound directly to a carrot cell. The binding of cellulose-minus mutants was generally weaker than the binding of wild-type bacteria and, although precautions were taken to avoid dissolving the bacteria during the experimental manipulations, this number represents a minimal estimate of the number of receptor sites on the surface of a carrot cell (6). However, observations in the light and scanning electron microscope suggest that the average carrot cell did not bind more than 500 cellulose-minus bacteria and that bacteria were relatively evenly distributed among the carrot cells. Thus, there appears to be a relatively small number of receptor sites for A. tumefaciens on the carrot cell surface. This result is in good agreement with the results of Neff and Binns (14) who, using a different technique, estimated that there are a few hundred receptor sites on tomato suspension culture cells.

It is not known whether each bacterium is bound via single or multiple molecular interactions at a receptor site. If only a small number of receptor proteins are involved in the binding of a single bacterium, then the polypeptides from the receptor site may be a minor component of the proteins eluted from the carrot cell surface. Calculations based on the number of carrot cells eluted and an average staining intensity for the receptor protein suggest that the darker bands on the electrophoresis gels represent polypeptides that are present in as many as 104 to 105 copies per carrot cell. Thus, the particular proteins involved in the carrot cell receptor for attachment of A. tumefaciens may be difficult to identify. However, the availability of elution techniques that remove the receptor and a treatment (embryo induction) which converts carrot cells which fail to bind the bacteria should aid further studies of the carrot cell receptor which binds A. tumefaciens.

Acknowledgments—The authors thank Dr. Mary Deasey for assistance with Figure 1 and Elaine Steele for assistance with Figure 5.

LITERATURE CITED

1. DAZZO FB, DH HURBELL 1975 Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl Microbiol 30: 1017–1033
16. OLD DC 1972 Inhibition of the interaction between fimbrial haemagglutinins and erythrocytes by D-mannose and other carbohydrates. J Gen Microbiol 71: 149–157