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Binding and Penetration of *Rhizobium Japonicum* to Cultured Soybean Cells

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A cultured soybean cell line, SB-1, was used to evaluate the initial interaction between the soybean cells and *Rhizobium japonicum*. Co-culturing of *R. japonicum* with SB-1 cells in suspension resulted in strain-specific polar attachment. This attachment can be inhibited by galactose and antibodies raised against seed soybean agglutinin (SBA). A lectin was purified from SB-1 cells which shares properties with SBA in terms of immunological reactivity, sugar binding activity, polypeptide molecular weight and peptide maps. When the SB-1 cells were co-cultured with *R. japonicum* for three weeks in solid agar medium, histological staining revealed bacterial penetration into certain SB-1 cells. Furthermore, there were focal regions of cells with prominent nuclei representing actively proliferating regions. These observations are analogous to that of *in vivo* nodule initiation in soybean roots.

Rhizobium japonicum binds specifically to the root hairs of its leguminous host, the soybean. The molecular basis for this recognition phenomenon appears to involve specific interactions between a lectin on the plant cell wall and a glycosylated ligand on the bacterial capsular surface [1, 2]. The lectin-recognition hypothesis has been questioned due to numerous inconsistencies and contradictions in data from various laboratories (for a review on the soybean system, see [3]). These inconsistencies in results are most probably due to the complexity of the experimental system utilizing whole plants or roots.

We have adopted a well defined soybean cell culture system to evaluate the mechanism for the initial interaction. In this report, we provide evidence supporting the lectin-recognition hypothesis. These studies also indicate that initial bacterial binding to the soybean cells leads to bacterial infection.

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Materials and Methods

Antibodies Raised Against Seed Soybean Agglutinin (SBA)

Seed SBA was purified from soybean meal (*Glycine max*) (Crop and Soil Department, Michigan State University, MI, USA) by affinity chromatography on Sepharose-*N*-ε-aminocaproyl-D-galactosamine columns [4]. This purified lectin was used to immunize New Zealand white rabbits (Bailey Farm, MI, USA). Monospecific anti-seed SBA antibodies were purified by affinity chromatography on an SBA-Sepharose column [5].

Soybean SB-1 Cell Line and SB-1 Lectin Isolation

The soybean SB-1 cell line, derived from soybean root (*Glycine max* [L]), was cultured in suspension with 1B5C medium as previously described [6].

The lectin synthesized by the SB-1 cells was designated as SB-1 lectin. This lectin could be isolated from three different sources:
(a) *Cell extract*: SB-1 cells were homogenized in a Waring blender for 5 min at maximum speed and then extracted with 0.5% sodium deoxycholate in 10 mM Tris-HCl, pH 7.4, overnight. After extraction, the cell extract was centrifuged at 10 000 × *g* for 30 min to remove cell debris. The supernatant fraction was used for lectin purification.
(b) *Cell wall fraction*: The cell wall of SB-1 cells was digested with cellulase (10 mg/ml) and pectinase (5 mg/ml) in 0.5 M sorbitol, pH 5.5, for 2 h at 37°C. The mixture was centrifuged at 10 000 × *g* for 30 min and the supernatant fraction was adjusted to pH 7.4 for lectin purification.
(c) *Culture medium*: The culture medium from 4-day-old SB-1 cells was collected by filtration through a Whatman No. 1 filter. The collected medium was adjusted to pH 7.4 and centrifuged (10 000 × *g*, 30 min). The supernatant fraction was used for lectin isolation.

Isolation of the SB-1 cell lectin was performed by affinity chromatography, the same as that used for seed SBA isolation [4]. The bound lectin was eluted from the Gal-Sepharose column by 0.2 M galactose in phosphate-buffered saline (PBS, 3 mM KCl, 0.137 mM NaCl, 10 mM sodium phosphate, pH 7.4).

Polyacrylamide Gel Electrophoresis, Peptide Map Analysis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was performed in 10% acrylamide gels. Immunoblotting analysis was carried out with anti-seed SBA antibody as primary antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase as secondary antibody (Sigma, St. Louis, MO, USA). The proteins reactive with anti-seed SBA antibody were revealed by peroxidase staining. Peptide maps of the seed SBA and the SB-1 lectin were generated by partial digestion with *Staphylococcus aureus* V8 protease [7].

Rhizobium Culture and Binding to SB-1 Cells

Various Rhizobium strains were cultured in liquid yeast extract-mannitol-gluconate medium at 30°C [8]. SB-1 cells (5×10^6 cells) in 1 ml suspensions were inoculated with 0.1 ml of Rhizobium culture (2.6×10^8 cells). The mixtures were incubated at 26°C for

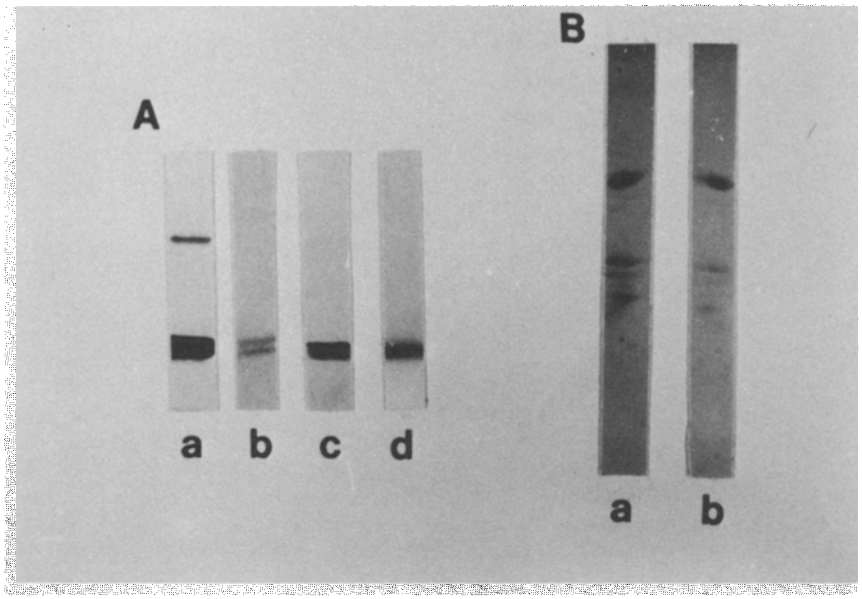

Figure 1. A. SDS-PAGE and immunoblotting analysis of purified lectin samples. (a) seed SBA; (b) lectin from deoxycholate extract of SB-1 cells; (c) lectin from digested cell wall fraction; and (d) lectin from culture medium.
B. Peptide maps of purified seed SBA (lane a) and SB-1 lectin from medium (lane b) after limited V8 protease hydrolysis as described by Cleveland *et al.* [7].

2-24 h in the dark. After incubation, the cells were washed three times with 1B5C medium by centrifugation and resuspension and examined under the microscope.

Histological Studies of SB-1 Cells Infected with Rhizobium

SB-1 callus cultures were grown in 1B5C medium with 0.8% agar. The callus was inoculated with 50 μl of various Rhizobium strains. After co-culture in the dark for 1 week at 26°C, the callus was transferred to another agar plate containing LNB5 medium with 0.8% agar [9]. The callus was further cultured for two weeks and then fixed, dehydrated and processed for sectioning. Sections were stained either with Gram stain or hematoxylin-eosin stain.

Results

Characterization of SB-1 Lectin Isolated from SB-1 Cells

The SB-1 lectin from SB-1 cells was purified from three different sources, all derived from cultured SB-1 cells, by affinity chromatography on Gal-Sepharose columns: (a) deoxycholate extract of SB-1 cells; (b) supernatant fraction after digestion of cell wall with cellulase and pectinase, and (c) the culture medium of 4 day suspension culture. Fig. 1A shows the immunoblotting analysis of these purified preparations as compared with

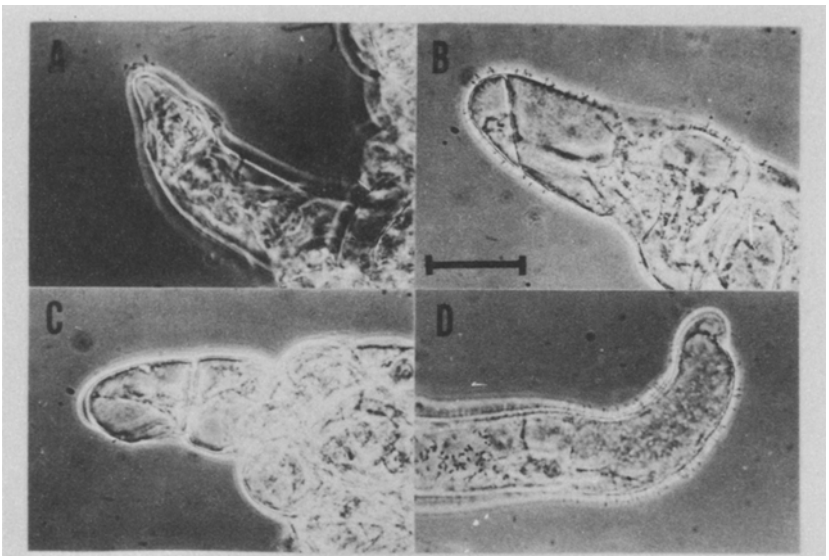

Figure 2. Adhesion of *Rhizobium japonicum* (R110d) to SB-1 cells after co-culture for (A) 2 h; and (B, C, and D) 24 h. (C) Co-culture in the presence of 20 μg/ml anti-seed SBA antibody; (D) co-culture in the presence of 1 mg/ml of anti-cell wall antibody. Bar = 10 μm.

seed SBA. The SB-1 lectin was identified by its cross-reactivity with anti-seed SBA antibody and was revealed as one predominant band (M_r 30 000), corresponding to that of the seed SBA.

Peptide maps of seed SBA and the SB-1 lectin purified from the medium were identical (Fig. 1B). When the purified SB-1 lectin (medium) was detected by radioimmunoassay [10], the position of elution of this component corresponded to a protein of molecular weight of 110 000, identical to the tetrameric form of seed SBA. These results suggest that SB-1 cells synthesize a lectin with carbohydrate binding activity, subunit structure, peptide maps and immunological properties similar to those of seed SBA. Furthermore, this lectin could be isolated from the cell wall fraction, a good source relative to deoxycholate extracts and culture medium collections. The presence of lectin in the cell wall, accessible to other molecules including anti-SBA antibody [5], is consistent with the possibility that it plays a role in Rhizobium recognition.

Rhizobium Binding to SB-1 Cells

When *Rhizobium japonicum* (R110d) was co-cultured with SB-1 cells at 26°C for 2 h, only a small number of SB-1 cells showed bacterial binding. The initial binding seems to be limited to the tips of some SB-1 cells (Fig. 2A). Prolonged incubation to 24 h resulted in specific attachment of the bacteria in a polar fashion (Fig. 2B).

The binding of Rhizobium to SB-1 cells was strain specific. Only the strains of Rhizobium that nodulate soybean, such as *R. japonicum* and *R. fredii*, showed binding. Other strains, such as *R. meliloti*, *R. trifolii* or an unrelated strain, *Escherichia coli*, did not show any binding.

Table 1. Carbohydrate specificity of Rhizobium binding.

Saccharide[a]	Inhibition of polar binding to SB-1 cells
Control	
Galactose[b]	+
N-Acetyl-galactosamine	—
Lactose[b]	+
Galacturonic acid[b]	+
Gluconic acid	—
Mannose	—
Glucose	—
Melibiose	—
Glucuronic acid	—
Xylose	—

[a] Saccharide concentrations varied from 3 mM to 0.2 M.
[b] At a saccharide concentration of 3 mM or above, polar binding of *Rhizobium* to SB-1 cells was inhibited.

A carbohydrate recognition system mediating the bacterial binding was suggested by specific saccharide inhibition. At concentrations as low as 3 mM, lactose, galactose or galacturonic acid showed complete inhibition of Rhizobium binding. Melibiose and other saccharides, including *N*-acetylgalactosamine, were not inhibitory (Table 1).

Further examination of the role of SB-1 lectin in Rhizobium binding was performed by blocking the binding with antibody raised against seed SBA. Anti-seed SBA antibody at a concentration of 20 μg/ml blocked Rhizobium binding (Fig. 2C), while normal rabbit immunoglobulin showed no effect. In order to show that an antibody binding to the cell wall components did not necessarily inhibit Rhizobium binding, an anti-cell wall antibody was generated by using a cell wall fraction as antigen. The cell wall fraction was obtained by extensive extraction of the SB-1 cells by SDS, methanol and water. This cell wall fraction retained the boundary of the ruptured cells, but no Rhizobium binding to these cell wall fragments was observed under the binding conditions. An antibody raised against this cell wall fraction showed antibody labelling on the cell wall by immunofluorescence suggesting that this antibody bound to the cell wall of SB-1 cells. Immunoblotting analysis indicated that this cell wall antibody showed no cross-reactivity to SBA. When this anti-cell wall antibody was incubated with Rhizobium and SB-1 cells, no blocking activity was found (Fig. 2D). Since only the anti-seed SBA antibody showed specific inhibition of Rhizobium binding, it is suggested that the lectin on the cell wall of SB-1 cells was possibly responsible for Rhizobium recognition.

Penetration of Rhizobium to SB-1 Cells

To test whether the binding of Rhizobium to the SB-1 cells would lead to penetration of the bacteria into the soybean cells, we examined SB-1 cells in callus culture that had been inoculated with *Rhizobium japonicum* for three weeks. Histological staining with Gram strain revealed the presence of bacteria within certain cells indicating bacterial infection of these cells (Fig. 3A). In addition, bacteria were observed in the interstitial

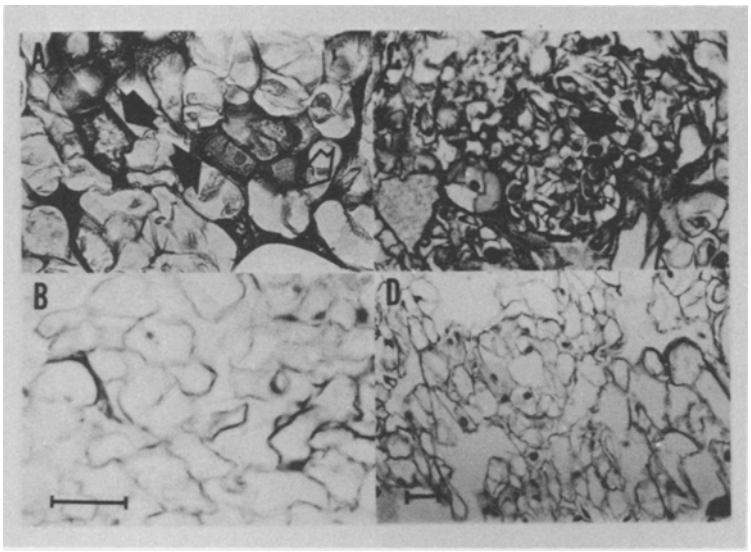

Figure 3. Histological staining of callus cultures of SB-1 cells with (A and C) or without (B and D) *Rhizobium japonicum* (R110d). A and B, SB-1 callus culture stained with Gram stain. The black arrow indicates infected cells. The open arrow shows bacteria in the interstitial space. (C and D) Callus cultures stained with hematoxylin-eosin. The black arrow highlights a focal region of proliferative cells. Bar = 10 μm.

space, a modified structure reminiscent of the pseudo-infection thread found in the root infected with Rhizobium [11]. Furthermore, focal regions of actively proliferated cells were observed after hematoxylin-eosin staining. These cells showed relatively more prominent nuclei (Fig. 3B). In contrast, control cells contained many areas that were not stained, representing large vacuoles inside the cells (Fig. 3D). These observations are similar to those reported previously on the *in vivo* infection of soybean roots by Rhizobium [12].

Discussion

The data documented in the present study indicate that incubation of *Rhizobium japonicum* wih the soybean SB-1 cell line results in polar adhesion of the bacteria to the soybean cells. This binding interaction appears to be strain specific. Only homologous Rhizobium strains that nodulate soybean roots show binding. Other strains of Rhizobium or an unrelated strain of gram negative bacteria, namely *E. coli*, showed no apparent adherence to the soybean cells. In spite of substantial evidence supporting host-specific Rhizobium binding to leguminous roots [13-15], there are other reports which indicate that nonspecific binding occurs between heterologous Rhizobium strains [16, 17]. This discrepancy of results may be due to various conditions and criteria employed for measuring bacterial attachment. Various time intervals for the assay can

be a major factor giving rise to different results. Dazzo *et al.* [13] have studied in detail the mechanism of *R. trifolii* attachment to clover roots. The results suggest that the initial phase of binding within 1 h of incubation may be reversible and nonspecific, while the later phase of interaction after 4-8 h incubation involves specific attachment of the bacteria to the root hair. Our present data show that significant binding of *R. japonicum* to SB-1 cells could be achieved after a prolonged culture time of 16 h. Furthermore, other heterologous bacteria failed to show any attachment at all. Therefore, this attachment phenomenon seems to be significant in controlling host specificity.

The Rhizobium-soybean cell association appears to be mediated via a cell wall component on the soybean root cells. Extraction of SB-1 cells with SDS, methanol and water resulted in a cell wall fraction that Rhizobium could no longer bind. Results from studies on the sugar inhibition of Rhizobium binding are consistent with a specific carbohydrate recognition mechanism. This recognition could be blocked by galactose, lactose or galacturonic acid, while glucose and other saccharides did not show any inhibitory effect.

A galactose-specific lectin on the cell wall of SB-1 cells most probably mediates the bacterial binding. This notion is supported by the fact that a lectin could be isolated from the digested cell wall fraction. Furthermore, rabbit anti-seed SBA antibody blocked the Rhizobium-soybean cell adhesion, while control normal rabbit immunoglobulin or anti-cell wall antibody showed no inhibitory effect. Characterization of this lectin showed similarities to seed SBA in terms of subunit molecular weight, immunological cross-reactivity, peptide maps and carbohydrate binding activity. It should be noted that *N*-acetylgalactosamine, a known hapten for seed SBA, did not inhibit Rhizobium binding to SB-1 cells. This may reflect a difference between the SB-1 lectin and seed SBA. However, SB-1 lectin bound to the Gal-Sepharose column could be eluted by 0.2 M *N*-acetylgalactosamine, as detected by immunoblotting analysis [10]. This result suggests that SB-1 lectin, in soluble form, shows the same carbohydrate binding specificity as that of seed SBA. Alternatively, it may reflect that the lectin anchored on the cell wall does not bind *N*-acetylgalactosamine.

Several observations suggested that this defined cell culture system mimics at least the early phase of the nodule formation in soybean roots. First, the binding of Rhizobium is polar as has been observed in the number of Rhizobium binding to the roots. Second, histological studies indicate the presence of bacteria within the intracellular space which suggest possible infection of certain cells. Third, the morphology of the intercellular structures, in which the bacteria migrate between cells, is reminiscent of the infection thread in the soybean root during the infection process. Fourth, staining with hematoxylin-eosin reveals that, upon co-culturing of the soybean cells with Rhizobium, there is a prominent increase in the size of the nucleus and cell proliferation, phenomena analogous to the tumorous growth during nodule formation in the root. This root cell culture may provide a valuable system to elucidate certain aspects of the complex mechanisms of nodule formation.

Acknowledgements

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References

1 Hamblin J, Kent SP (1973) Nature 245:28-30.
2 Bohlool BB, Schmidt EL (1974) Science 185:269-71.
3 Pueppke SG (1983) in Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins, eds. Goldstein IJ, Etzler ME, Alan R Liss, New York, p 225-36.
4 Allen AK, Neuberger A (1975) FEBS Lett 50:362-64.
5 Ho S-C, Malek-Hedayat S, Wang JL, Schindler M (1986) J Cell Biol 103:1043-54.
6 Metcalf TN, Wang JL, Schubert KR, Schindler M (1983) Biochemistry 22:3969-75.
7 Cleveland DW, Fisher SG, Kirschner MW, Laemmli UK (1977) J Biol Chem 252:1102-6.
8 Bhuvaneswari TV, Turgeon BG, Bauer WD (1980) Plant Physiol 66:1027-31.
9 Child JJ, LaRue TA (1974) Plant Physiol 53:88-90.
10 Malek-Hedayat S, Meiners SA, Metcalf TN, Schindler M, Wang JL, Ho S-C (1987) J Biol Chem 262:7825-30.
11 Holston RD, Burns RC, Hardy RWF, Hebert RR (1971) Nature 232:173-76.
12 Dart P (1977) in A Treatise on Dinitrogen Fixation, eds. Hardy RWF, Silver WS, Wiley, New York, p 367-472.
13 Dazzo FB, Truchet GL (1983) J Membr Biol 73:1-16.
14 Stacey G, Paau AS, Brill WG (1980) Plant Physiol 66:609-14.
15 Kato G, Maruyama Y, Nakamura M (1981) Plant Cell Physiol 22:759-71.
16 Mills KK, Bauer WD (1985) J Cell Sci Suppl 2:333-45.
17 Pueppke SG (1984) Plant Physiol 75:924-28.