Effects of Boron on \textit{Rhizobium}-Legume Cell-Surface Interactions and Nodule Development$^1$

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Boron (B) is an essential micronutrient for the development of nitrogen-fixing root nodules in pea (\textit{Pisum sativum}). By using monoclonal antibodies that recognize specific glycoconjugate components implicated in legume root-nodule development, we investigated the effects of low B on the formation of infection threads and the colonization of pea nodules by \textit{Rhizobium leguminosarum} by \textit{vicieae}. In B-deficient nodules the proportion of infected host cells was much lower than in nodules from plants supplied with normal quantities of B. Moreover, the host cells often developed enlarged and abnormally shaped infection threads that frequently burst, releasing bacteria into damaged host cells. There was also an overproduction of plant matrix material in which the rhizobial cells were embedded during their progression through the infection thread. Furthermore, in a series of in vitro binding studies, we demonstrated that the presence of B can change the affinity with which the bacterial cell surface interacts with the peribacteroid membrane glyocalyx relative to its interaction with intercellular plant matrix glycoprotein. From these observations we suggest that B plays an important role in mediating cell-surface interactions that lead to endocytosis of rhizobia by host cells and hence to the correct establishment of the symbiosis between pea and \textit{Rhizobium}.

B plays an essential role in nodule development. Brenchley and Thornton (1925) described ineffective nodules in B-deficient \textit{Vicia faba}, and more recently we reported that pea (\textit{Pisum sativum}) nodules that developed under low-B conditions were not functional and became prematurely senescent (Bolaños et al., 1994). Three- and 4-week-old B-deprived nodules showed a generalized degeneration of cell walls and membranes, including the PBM, which surrounds intracellular bacteroids. Because of these impairments in nodule development, rhizobia inside nodules showed little or no ability to fix N$_2$. This leads to N$_2$ deficiency and to the necrosis of nodulated pea plants (Bolaños et al., 1994).

The physiological role of B has been related to the chemistry of boron ions: boric acid acts as a Lewis acid and forms anions by accepting hydroxyl ions. The special structure of boron anions makes them able to become esterified with cis-diol groups from polyhydroxy compounds (Mazurek and Perlin, 1963), most notably in carbohydrate molecules. Thus, B seems to be responsible for the stabilization of glycoconjugates in plant cell walls (Loomis and Durst, 1991) and membranes (Parr and Loughman, 1983). Similarly, B has been shown to be essential in maintaining the structural integrity of specialized carbohydrate-rich structures present in the envelope of the N$_2$-fixing heterocyst of cyanobacteria (García-González et al., 1991).

During legume root-nodule development, plant-derived glycoconjugates or the glyco-components from the cell surface of \textit{Rhizobium} are thought to play an essential role in the correct establishment of the symbiosis between legumes and rhizobia (Kannenberg and Brewin, 1994, and refs. therein). Evidence from genetic analysis shows that colonization of host tissues and cells by \textit{Rhizobium} requires appropriate extracellular polysaccharide and LPS (Stacey et al., 1991). In peas and other legumes with indeterminate nodules \textit{Rhizobium} infection occurs through the formation of a transcellular tunnel-like structure, termed the infection thread, which grows through the cytoplasm by apical deposition of primary cell-wall material (Rae et al., 1992; van Spronson et al., 1994). During their growth and multiplication inside the thread, rhizobia are embedded in plant-derived intercellular material, including a MGP (Vanden-Bosch et al., 1989). Proximal to the meristematic zone of the nodule is an invasion zone where infection threads ramify and the bacteria begin to invade cells from unwalled infection droplets. When bacterial cells come into close contact with naked plant membrane, there is apparently a physical interaction between these two surfaces (Bradley et al., 1986), and bacteria are released into nodule tissue cells by endocytosis. The released bacteria (bacteroids) come to occupy an organelle-like compartment termed the symbiosome. These bacteroids continue to proliferate for some time in the host cytoplasmic space, and eventually they develop the capacity for N$_2$ fixation (Brewin, 1991). At all later stages during nodule development each bacteroid is individually surrounded by a plant-derived PBM that carries a differentiated form of plasma membrane glycoconalxy composed of a mixture of glycolipids and glycoproteins (Perotto et al., 1991).

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\textbf{Abbreviations:} LPS, lipopolysaccharide; MGP, matrix glycoprotein; PBM, peribacteroid membrane; TBS, Tris-buffered saline.
Because all of these stages in nodule development apparently involve some kind of interactions between plant-derived and bacterial cell-surface glycoconjugates, we investigated the effects of B deprivation on the processes of infection thread development and bacterial release by making use of a variety of monoclonal antibodies that recognize plant and bacterial cell-surface components. The same antibody probes were used for in vitro experiments to investigate the attachment of plant cell-surface components to free-living bacterial cells. During the course of these experiments on plant-Rhizobium cell-surface interactions, an effect of borate ion was identified that could help to explain the essential role of B during the development of pea nodules.

**MATERIALS AND METHODS**

**Plant Growth and Inoculation**

Pea (*Pisum sativum* cv Argona) seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 10% (v/v) sodium hypochlorite for 20 min, soaked for 4 h in sterile distilled water, and then germinated on wet filter paper at 25°C. After 7 d seedlings were transferred to plastic growth pots and cultivated on B-free perlite with Fahraeus-Plant pot medium for legumes (Fahraeus, 1957). Pea plants were inoculated with 1 mL per seedling of about 10^6 cells mL^(-1) of *Rhizobium leguminosarum* bv *viciae* 3841 from an exponential culture in tryptone-yeast extract medium (Beringer, 1974). Plants were maintained in a growth cabinet at 22°C day/18°C night with a 16-h/8-h photoperiod and an irradiance of 190 pmol m^(-2) s^(-1). RH was kept between 60 and 70%.

For B-free cultures, B was removed from the micronutrient solution. For cultures with the normal content of B, the micronutrient (as H$_2$BO$_3$) was added to a final concentration of 0.1 mg L^(-1) B. All solutions were prepared and stored in polyethylene containers previously demonstrated not to release B even under sterilizing conditions (Mateo et al., 1986). B was determined in the solutions and media for 30 min at 100,000g to remove debris. The resulting nodule supernatant was used in experiments as the source of MGP.

Pea nodules were fixed for 16 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Samples were dehydrated in an ethanol series and embedded in LR White resin (Rae et al., 1991). Semithin (0.5 µm) sections of nodules were counterstained with basic fuchsin or processed for immunogold staining and silver enhancement according to the methods previously described (Perotto et al., 1991). The secondary antibody was goat anti-rat IgG conjugated to 10-nm colloidal gold (Amersham).

**Fractionation of Nodules**

Pea nodules were homogenized at 4°C in Tris-DTT buffer (50 mM Tris-HCl, pH 7.5, 10 mM DTT) containing 0.5 M Suc. The soluble fraction of the homogenate was collected by filtration through Miracloth (Calbiochem) and centrifuged for 30 min at 100,000g to remove debris. The resulting nodule supernatant was used in experiments as the source of MGP.

**MGP-Rhizobium Incubations and Dot Immunoassays**

For cell-attachment studies, the nodule supernatant fraction (containing the MGP) derived from nodule homogenate was diluted 1:10 in TBS (50 mM Tris-HCl, pH 7.4, 200 mM NaCl) buffer. Aliquots (200 µL) were incubated with 50 µL of a suspension of bacterial cells (10^8 cells mL^(-1)) derived from a culture of *R. leguminosarum* 3841 grown for 2 d on a slant of tryptone-yeast extract medium agar and washed several times with TBS before use. After incubation for 30 min, either in the presence or in the absence of B as Borax (brand name of sodium tetraborate), the mixture was centrifuged for 1 min at 10,000g. After the sample was centrifuged the pellets from these incubations were washed three times in TBS and finally resuspended in 250 µL of TBS. Aliquots (1 µL) derived from pellet and supernatant fractions were dotted on nitrocellulose sheets and probed with the monoclonal antibody MAC 265 to test for MGP-binding activity or with MAC 57 to test for the presence of bacterial antigens. A goat anti-rat IgG conjugated to peroxidase (Amersham, UK) was used as a secondary antibody.

**Rhizobium-PBM Incubations and Dot Immunoassays**

Aliquots of the nodule-derived PBM fraction (5 µL containing approximately 2 µg of protein) were incubated for 1 h at 4°C with 50 µL of bacterial suspension or with equivalent volumes from the resuspensions of centrifuged pellets derived from the MGP-Rhizobium interaction exper-

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**Table 1. Rat monoclonal antibodies used in this study**

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<tr>
<th>Designation</th>
<th>Isotype</th>
<th>Antigen Recognized</th>
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<tbody>
<tr>
<td>AFRC MAC 265</td>
<td>Rat IgG$_{2a}$</td>
<td>MGP</td>
</tr>
<tr>
<td>AFRC MAC 57</td>
<td>Rat IgM</td>
<td>LPS (O-antigen) of <em>R. leguminosarum</em> 3841</td>
</tr>
<tr>
<td>AFRC MAC 206</td>
<td>Rat IgG$_{2c}$</td>
<td>Plant membrane glycolipid</td>
</tr>
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ment described above. After the samples were centrifuged for 2 min at 10,000g the resulting pellets were washed in TBS and resuspended in the same volume of buffer. Aliqouts (1 μL) were dotted onto sheets of nitrocellulose and probed with the monoclonal antibody MAC 206, which recognizes a plant membrane glycolipid antigen, or with MAC 57, which recognizes bacterial LPS. When appropriated, the resuspended pellets and supernatants resulting from these incubations were treated with 0.1% (v/v) (Nonidet P-40) as a surfactant. A goat anti-rat IgG conjugated to peroxidase was used as a secondary antibody.

RESULTS

As described in a previous study (Bolaños et al., 1994), we found that inoculated pea seedlings grown in the absence of B (B-minus plants) yielded root nodules that were smaller than for B-plus plants. The onset of nitrogenase activity of these B-minus nodules also seemed to be delayed and nitrogenase activity was lower in B-deprived nodules than in nodules of comparable age developed in the presence of B. In addition, most nodules developed in the absence of B still appeared white when examined 3 to 4 weeks after inoculation, whereas seedlings grown in the presence of B already had pink nodules. All of these observations apparently confirmed the essential role of B in nodule development that had been previously described (Brenchley and Thornton, 1925).

To reveal differences in tissue invasion between pea nodules developed in the presence or in the absence of B, longitudinal sections of young nodules (10 d after inoculation) were immunolabeled with the monoclonal antibody MAC 206, which recognizes a plant glycolipid on the PBM that encloses the rhizobia inside plant cells (Perotto et al., 1991). In control (B-plus) nodules, a large proportion of cells of the central nodule tissue was colonized by bacteria. Furthermore, positive immunostaining with MAC 206 revealed the presence of glycolipid antigens associated with PBM for the bacteria localized within the cytoplasm of host cells (Fig. 1A). In B-deficient nodules, however, immunostaining with MAC 206 revealed that only a few cells were colonized by bacteria enclosed within symbiosome compartments (Fig. 1B). These observations suggest that the poor development of nodules on pea seedlings grown under B-limiting conditions was associated with an impairment of tissue and cell invasion by rhizobia.

Effects of B Deficiency on Infection Thread Development

To study the process of cell and tissue invasion by *Rhizobium*, sections were taken from the invasion zone of nodules developed in the presence or in the absence of B. Sections were immunolabeled with the monoclonal antibody MAC 265, which recognizes the intercellular plant MGP secreted by plant cells into infection threads, infection droplets, and intercellular spaces (VandenBosch et al., 1989; Rae et al., 1991). Figure 2A shows the normal infection threads and droplets in B-plus nodules, with the presence of MGP revealed as a black image after silver enhancement of immunogold-stained sections. In B-deficient

nODULES immunostained with MAC 265 (Fig. 2, B and C), large invasion structures sometimes appeared in the central tissue of the nodule (arrow). These invasion structures contained MGP (MAC 265 antigen), and the presence of *Rhizobium* bacteria was verified by immunolabeling with MAC 57, an LPS-specific monoclonal antibody (Fig. 2D). Furthermore, the absence of PBM surrounding these rhizobial cells was indicated by the absence of immunostaining with MAC 206 (Fig. 2E), and this observation confirmed that the bacteria were embedded in intercellular matrix material rather than being intracellular.

In older nodules (2 or 3 weeks after inoculation), the enlarged infection structures found in nodules formed under B-deficient conditions extended even to the apical region of the nodule (data not shown). The typical irregular shape and fragility of B-deficient walls (Cohen and Lepper, 1977) was a general feature of older nodules. It was also possible to find abnormal bacterial release from these infection structures, possibly due to wall and membrane breaks, which occur very commonly in B-deficient nodules, as we previously reported (Bolaños et al., 1994).

Effects of B on Bacterial Cell-Surface Interactions

The effects of borate on the interaction among bacteria, plant matrix material, and PBM were tested in vitro by using nodule-derived extracts, including the MGP and PBM fractions obtained from pea nodules, together with free-living cells of *R. leguminosarum* 3841. After free-living bacteria and MGP were mixed, a binding activity of the MGP to the bacterial cell surface was detected after centrifuging the incubation mixture followed by immunolabeling the pelleted bacteria with MAC 265. The positive staining with MAC 265 illustrated in Figure 3 indicates that MGP is able to interact physically with the cell surface of *Rhizobium*. However, the inclusion of B (10 mm Borax) in the incubation mixtures inhibited the binding of MGP to bacteria (Fig. 3).

The interaction of free-living bacterial cells with isolated PBMs was also studied in vitro using MAC 206 as a probe, which recognizes a glycolipid component of the plasma membrane and PBM. As expected, MAC 206 antibody recognized the PBM in dot immunoassays when no bacteria were present in the incubation mixture (Fig. 4A, MAC 206 probe): after low-speed centrifugation some of this antigen was detected in the pellet and some was detected in the supernatant fraction. However, after incubation with salt-washed bacterial cells derived from a free-living culture of *Rhizobium*, an apparent net loss of MAC 206 antigen (PBM-glycolipid) was observed both in the pellet and in the supernatant fractions (Fig. 4B). The loss of detectable antigen from both fractions was somewhat surprising and was interpreted to be due to the occlusion of PBM glycolipids brought about by its binding to the bacterial cell surface. This possibility was supported by the observation that after treating the pellets with 0.1% (v/v) Nonidet P-40 as a surfactant a reappearance of epitope recognized by MAC 206 was detected in the released supernatant fraction (Fig. 4, B' and C'). The occlusion of MAC 206 antigen by tight association with bacteria in the pellet fraction was further
Figure 1. Comparison of pea nodule tissue sections taken from plants grown with or without B and sampled 10 d after inoculation with *R. leguminosarum* 3841. Sections were stained with MAC 206 antibody, which recognizes a glycocalyx antigen present on plasma and PBMs. Following immunogold labeling and silver enhancement, the sections were not counterstained, so that the black image represents the distribution of MAC 206 antigen, i.e. the distribution of plant cells and PBMs and in particular the presence of symbiosomes in the infected region (arrow). A, Nodule from a plant grown with B (0.1 mg B L$^{-1}$); B, nodule from a plant grown without B. m: Nodule meristem. Bar marker represents 0.1 mm.
Figure 2. Sections derived from the invasion tissues of pea nodules 10 d after inoculation with *R. leguminosarum* 3841 showing infection structures (black images highlighted by arrowheads) developed in the presence (A) or in the absence (B–E) of B. C, D, and E are serial sections from the same segment of tissue highlighted by an arrow in B. A, B, and C have been immunostained with MAC 265 and silver enhanced to show the presence of the plant MGP, which is overproduced in the absence of B; D has been immunostained with MAC 57 to show the presence of *R. leguminosarum* 3841 inside the infection structures formed in B-deficient conditions; E has been immunostained with MAC 206 to confirm the absence of plant membrane material surrounding bacteria inside the infection structure. m, Nodule meristem. Bar markers represent 0.1 mm (A and B) and 10 μm (C–E).
Figure 3. Dot immunoassays showing the effect of borate on in vitro binding of the plant intercellular MGP to cells of *R. leguminosarum* 3841. Free-living bacteria were incubated with the soluble fraction of pea nodules, containing the MGP, and B was added as Borax (sodium tetraborate). After the samples were centrifuged, the pellets from these incubations were resuspended and aliquots (1 μL) were dotted on nitrocellulose sheets and probed with the monoclonal antibody MAC 265 (anti-MGP) to test for binding activity. Replicate nitrocellulose sheets were incubated with MAC 57 (anti-LPS) as a control for loading bacterial cells. Both sheets were immunostained with anti-rat immunoglobulin-peroxidase conjugate followed by a chromogenic substrate.

confirmed by SDS-PAGE and western blotting of material derived from the pellet fraction (data not shown). The presence of borate in the incubation mixture had no effect on the interaction of bacterial cells with PBM, which resulted in the apparent occlusion of MAC 206 antigen (Fig. 4, C and D). Although B did not have a direct effect on the interaction between plant membrane and *Rhizobium*, an indirect effect was observed in the case of bacteria that had already been pretreated with MGP. In the absence of B, it was shown that when rhizobial cells were pretreated with nodule extracts containing MGP and subsequently challenged with PBM fractions (Fig. 5) there was apparently no interaction with PBM (because no occlusion of MAC 206 antigen was observed). However, when B was present in the original incubations, it inhibited the interaction between MGP and rhizobia, and the bacterial surface was subsequently able to interact with PBM glycolcalyx and thereby occlude MAC 206 antigen.

**DISCUSSION**

B has commonly been implicated as an important component of glycoconjugate-rich structures, e.g. the plant cell wall (Loomis and Durst, 1991), the plant plasma membrane (Parr and Loughman, 1983), and the heterocyst envelope of cyanobacteria (Garcia-González et al., 1991). In legume nodules B also apparently plays a structural role by maintaining the integrity of cell wall and membranes (Bolaños et al., 1994).

Figure 1 shows that the first symptoms of B deficiency on nodule development appeared approximately 10 d after inoculation. Although B was removed from the nutrient solution for pea germination and growth, a small amount of B was detected in B-minus nodules (3.87 ± 0.23 μg B g⁻¹ dry weight, compared to 43.53 ± 3.45 μg B g⁻¹ dry weight for control nodules derived from peas grown in the presence of B). Presumably, the B detected in B-minus nodules originated from reserves present in the seed. B has been shown to be a structural component of glycoconjugate-rich structures in several systems, including legume nodules (Loomis and Durst, 1991), plant plasma membranes (Parr and Loughman, 1983), and bacterial membranes (Garcia-González et al., 1991). In legume nodules B also appears to play a structural role in maintaining the integrity of cell wall and membranes (Bolaños et al., 1994).

Figure 4. Dot immunoassays showing the effects of borate on in vitro binding of PBM fractions (derived from pea nodules) to *R. leguminosarum* 3841 cells. Suspensions of free-living bacteria were incubated (1 h, 4°C) with PBM fractions and borate was added as Borax (sodium tetraborate). After incubation and centrifugation, both the resuspended pellet (P) and supernatant (S) fractions were dotted (1 μL) on nitrocellulose sheets and probed with the monoclonal antibody MAC 206 (anti-PBM-glycolipid). Replicate nitrocellulose sheets were incubated with MAC 57 as a control for loading bacterial cells. A, No bacteria and no borate were added (note that, because PBMs were isolated from pea nodules harboring strain 3841, a background of 3841 IPS is detected by MAC 57); B, bacteria but no borate were added; C, bacteria and 10 mM borate were added; D, bacteria and 50 mM borate were added. Pellets derived from incubations of bacteria and membrane fractions in the absence or presence of borate were resuspended in Nonidet P-40 (0.1% in TBS). After the samples were centrifuged a second time, the new pellets and supernatants were dotted on nitrocellulose sheets and probed with MAC 206. A', Material derived from pellet A; B', material derived from pellet B; C', material derived from pellet C. Reappearance of MAC 206 labeling in the supernatant sheets indicates release of MAC 206 antigen derived from membrane previously bound to the bacterial surface in a form that was inaccessible to MAC 206 antibody (B' and C'). All of the sheets were immunostained with anti-rat immunoglobulin-peroxidase conjugate.
endocytosis. Furthermore, results shown in Figure 5 suggest that the binding of MGP to Rhizobium (in the absence of B) could block the interaction between bacterial cell surfaces and plant membrane antigens. The absence of B could block the interaction between bacterial cell surfaces and plant membrane antigens. It is therefore possible that during nodule development in the absence of B the process of endocytosis is impaired in a similar fashion.

According to these results and interpretations, we suggest a possible model describing the role of B in nodule invasion. In the presence of B, Rhizobium could progress through the infection thread, without interacting with the MGP (Fig. 6, 10 mM borate line), toward an infection droplet, where the bacteria are probably unencapsulated. As a result of bacteria-plant cell-surface interactions (Bradley et al., 1986), bacteria are individually engulfed by the plant cell membrane, causing bacterial release into the host cell by endocytosis (Fig. 6, 10 mM borate line). In the absence of B, Rhizobium would be “trapped” by the MGP when the bacteria discontinue the synthesis and secretion of a capsule of extracellular polysaccharide in the region of the infection droplet (Fig. 6, no borate line). Under these conditions, rhizobia would be unable to interact with the plant membrane. Therefore, according to this model, endocytosis and bacterial release would be prevented in the absence of B.

B could also play an essential role during bacteroid differentiation. The absence of B seems to destabilize the glycoconjugate components of the inner face of the PBM (data not shown). During the stage of most active N₂ fixation in normal conditions, nitrogenase activity in B-deprived nodules was reduced to about 20%. Symbiosomes appeared with a very abnormal morphology and usually contained several bacteroids. Bacteroids inside them appeared with a very abnormal morphology and usually contained several bacteroids. Bacteroids inside them appeared with a very abnormal morphology and usually contained several bacteroids. Bacteroids inside them appear...
peared very stressed, indicating that they could not adjust to the microenvironmental conditions of the symbiosome compartment (Bolaños et al., 1994). These observations suggest that the integrity of the glycocalyx is of critical importance in the differentiation of N$_2$-fixing bacteroids. Furthermore, observations in nodules induced by LPS-defective mutants (Perotto et al., 1994) suggest that there may be an interaction between the plant membrane glycocalyx and the bacterial cell surface, as we have shown by in vitro experiments.

Investigating the effects of B in nodule development could shed new light on the role of some plant-derived and bacterial cell-surface glycoconjugates in this process. Many cases have been described in which the host-Rhizobium interaction fails to develop into a normal N$_2$-fixing symbiosis (Priefier, 1989; Niehaus et al., 1993; Perotto et al., 1994) because mutations affecting bacterial surface components result in a mild form of host defense response that is characterized by irregularities of infection thread development and a reduced level of colonization of host cells. Now we show that the anatomy of nodules developed in the absence of B has similarities with that of nodules induced under normal growth conditions by LPS-defective Rhizobium mutants, suggesting that the presence of B could suppress a mild form of plant defense response similar to that described for nodules occupied by mutants of Rhizobium with defective cell-surface properties (Perotto et al., 1994). Similarly, B could be needed for the correct progression of rhizobia through the infection thread and for the release of bacteria into host cells by bacterial endocytosis. Furthermore, by stabilizing the plant membrane glycocalyx, B could strengthen the physical interaction between the PBM and bacteroids, which seems to be very important in establishing the nutritional relation between legumes and rhizobia that leads to the N$_2$-fixing symbiosis.  

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Brenchley WE, Thornton HG (1925) The relation between the development, structure and functioning of the nodules on Vicia faba, as influenced by the presence or absence of boron in the nutrient medium. Proc R Soc Lond B Biol Sci 98: 373–398  