

Expression of Extracellular Glycoproteins in the Uninfected Cells of Developing Pea Nodule Tissue

A. L. Rae, S. Perotto, J. P. Knox, E. L. Kannenberg, and N. J. Brewin

John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.
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Two monoclonal antibodies, AFRCMAC265 and JIM13, with specificity for plant extracellular glycoproteins were used to study the organization and development of the uninfected tissue layers in nodules induced on the roots of *Pisum sativum* by *Rhizobium leguminosarum* bv. *viciae*. A 95-kD glycoprotein, recognized by MAC265 and previously identified in the matrix of infection threads, was detected in the intercellular spaces of the uninfected nodule parenchyma and also in the uninfected tissue in the vicinity of the apical meristem. It was also found in the intercellular spaces of empty nodule-like structures induced by a mutant of *R. leguminosarum* that has a defective lipopolysaccharide structure.

Additional keywords: immunogold labeling, legume root nodule.

Rhizobium and *Bradyrhizobium* spp. induce nitrogen-fixing nodules on the roots of leguminous plants (Long 1989). As a result of the exchange of signal molecules between plant and microbe, the normal developmental pathways of the root are subverted to create an entirely new organ. Despite the diversity of nodule morphology (Sprent 1989), four anatomical features seem to be common for all legume nodules: induction of a new plant meristem within the root cortex; tissue and cell invasion by *Rhizobium*; development of a microaerobic central tissue within which nitrogen fixation can take place; and development of a peripheral vascular tissue external to the central infected tissue, but inside the sheath of the nodule endodermis, which encloses the central tissues of the nodule (Robertson and Farnden 1980).

In pea, alfalfa, clover, and vetch, the root nodules are cylindrical in shape with an uninfected apical meristem that differentiates proximally into both infected and uninfected cell types (Fig. 1). Because of the persistent (indeterminate) nature of the pea nodule meristem, all stages of cell and tissue development can be observed in a single median longitudinal section (VandenBosch *et al.* 1989b, Nap and Bisseling 1990). The central infected tissue is surrounded by the uninfected nodule parenchyma (inner cortex), a shell of relatively undifferentiated cells that is traversed longitudinally by small vascular bundles (Van de Wiel *et al.* 1990b). The uninfected nodule parenchyma

Enhanced secretion of this glycoprotein is thus a very early response to *Rhizobium* infection. A different class of glycoprotein, recognized by JIM13, was localized specifically to cell walls in the endodermal sheath of pea nodules. JIM13 identified differentiating endodermal cells before wall thickening was apparent, and also labeled cytoplasmic vesicles in postmeristematic uninfected cells. JIM13 also identified the incipient endodermis in empty nodule-like structures. We conclude that the expression and secretion of glycoproteins is related to the early differentiation of distinct cell types in the uninfected tissues of the nodule.

is bounded by a single layer of endodermal cells, which forms a sheath that is continuous with the root endodermis but does not extend into the apical meristematic zone. External to the endodermis is a second parenchymatous layer, the outer cortex.

Specific molecular probes have been used to study the organization and development of nodule tissues and the associated patterns of gene expression. These probes in-

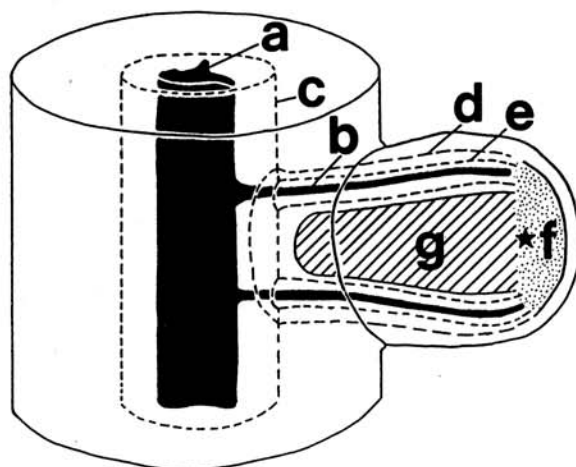


Fig. 1. Diagram showing the major tissue layers and cell types in a pea nodule (adapted from Bond 1948): A, root xylem; B, nodule xylem strand; C, root endodermis; D, nodule endodermis; E, nodule vascular endodermis; F, nodule meristem; G, central infected tissue; * indicates the "invasion zone" where infection threads develop in postmeristematic uninfected cells. The uninfected nodule parenchyma is defined as the region situated between the central infected tissue (G) and the nodule endodermis (D), which forms a sheath around the central tissue.

Present address of J. P. Knox: Centre for Plant Biochemistry and Biotechnology, University of Leeds, Leeds LS2 9JT, UK.

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clude antisera to nodule-specific proteins such as leghemoglobin (Robertson *et al.* 1984) and uricase (VandenBosch and Newcomb 1986), and antisense cDNA probes that bind to nodule-specific gene transcripts (Scheres *et al.* 1990 a,b). These studies of nodule development have largely focused on the central infected zone, but the uninfected outer layers are clearly also important, both for the organization of nodule development (Van de Wiel *et al.* 1990b) and for the regulation of oxygen, nitrogen, and carbon exchanges to the central tissues of mature nodules (Sprent 1989; Witty *et al.* 1986). Here we describe the use of monoclonal antibodies as molecular probes to recognize epitopes on distinct extracellular glycoproteins specific to two adjacent tissue layers in the uninfected region of the pea nodule. One of the glycoproteins was expressed by cells of the uninfected nodule parenchyma and the other was localized to cell surfaces of the endodermal sheath.

MATERIALS AND METHODS

Biological materials. Peas (*Pisum sativum* L. 'Wisconsin Perfection') were grown as previously described (Brewin *et al.* 1983) and inoculated with *Rhizobium leguminosarum* bv. *viciae* Jordan strain 3841 (Brewin *et al.* 1985), or mutant strain B659. Strain B659 was selected after mutagenesis of strain 3841 with the transposon Tn5, using as donor

Escherichia coli S17-1 pSUP 1021 (Simon *et al.* 1986; Wood *et al.* 1989). The mutagenized strain was plated on complex medium (TY) containing 0.6 mM CaCl₂ and selective antibiotics. Strain B659 has a characteristic slime-reduced colony morphology and abnormalities in the bacterial lipopolysaccharide (LPS) and exopolysaccharide (E. L. Kannerberg, in preparation). It induces small white nodules on peas, with no detectable capacity for acetylene reduction (nitrogen fixation). Immunostaining of these nodules suggested that they did not contain bacteria or infection threads: MAC57, an LPS-specific monoclonal antibody (Brewin *et al.* 1985), failed to identify any bacteria within the nodule structure, and similarly it was impossible to detect intracellular infection threads by staining for matrix glycoprotein with MAC265 antibody (VandenBosch *et al.* 1989a).

Antibodies. AFRCMAC265 is a rat monoclonal antibody described previously (VandenBosch *et al.* 1989a) and thought to recognize an epitope on a 95-kD extracellular matrix glycoprotein. JIM13 is a rat monoclonal antibody (class IgM) that was derived, using the IR983F myeloma (Bazin 1982), after immunization with high molecular weight glycoproteins from the conditioned medium of a carrot cell suspension culture. In that system, JIM13 recognizes a proteoglycan of the arabinogalactan protein (AGP) class and was selected for its developmentally regu-

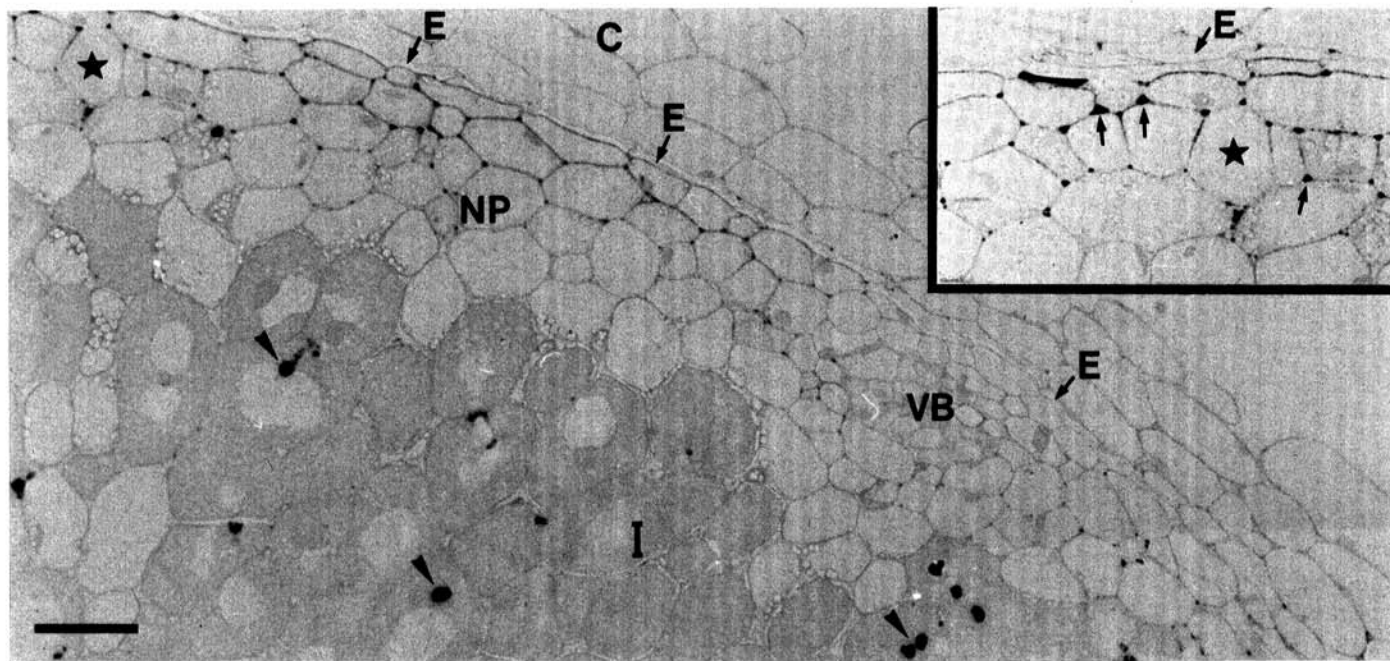


Fig. 2. Light micrographs of a median longitudinal section through a pea nodule after immunogold labeling with MAC265 antibody followed by silver enhancement. Bar represents 0.1 mm. Black deposits of silver show the presence of the MAC265 antigen against a grey background that is due to lightly counterstaining with basic fuchsin. This section was taken from the same nodule as shown in Figure 4, and a comparison of the different antigen labeling patterns provides a control for the specificity of the two antibodies used. The nodule apex is to the lower right of this field of view, which includes the outer cortex (C), endodermis (E), nodule parenchyma (NP) containing a vascular bundle (VB), and the central infected tissue (I). MAC265 antigen is concentrated in the intercellular spaces of the nodule parenchyma. Inset shows an enlarged view of a region of the nodule parenchyma to the upper left of the main micrograph. The same cell is marked by a star in both views. Examples of labeling in the intercellular spaces are marked with arrows in the inset. In the central infected tissue of the nodule, MAC265 antigen is concentrated in infection droplets (examples are indicated by arrowheads in the main micrograph); these contain undifferentiated bacteria and arise from tubular infection threads (not shown). In the absence of counterstaining, the image is absolutely blank, apart from the black silver deposits identified by arrowheads in the main micrograph and by arrows in the inset.

lated pattern of labeling in the carrot root apex (Knox *et al.* 1989, 1991).

Immunolabeling. Pea nodules were fixed overnight at 4° C in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Samples were dehydrated in an ethanol series and embedded in LR White resin at -20° C as previously described (VandenBosch *et al.* 1989b). Tissue sections for light microscopy (0.5–1 µm) or for electron microscopy (80 nm) were immunolabeled and stained by the methods of VandenBosch *et al.* (1989b). Janssen "Auroprobe" colloidal gold reagents and IntenSE "M" silver enhancement kits were obtained from Cambio (Cambridge, UK).

Four kinds of experiments were included to verify the specificity of the antibodies used in immunolabeling: western blots of nodule material identified the antigens biochemically; with no first antibody or with an irrelevant first antibody, MAC83 (VandenBosch *et al.* 1989a), the result after silver staining was always an absolutely blank image for all nodule sections; throughout this study, two antibodies with different specificities were compared (MAC265 and JIM13) and the discrete localization pattern obtained with each antibody served as a specificity control for the other antibody; to verify that black deposits visualized after silver enhancement reflected the specific accumulation of antigen, regions of tissue identified in the light microscope studies were subsequently examined by electron microscopy after immunogold staining.

Gel electrophoresis and immunoblotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) using 12% acrylamide mini-gels. Proteins were transferred electrophoretically to nitrocellulose sheets (Schleicher and Schuell, Dassell, Germany) for 16 hr at 10 V in 25 mM sodium phosphate buffer, pH 6.5, using a Bio-Rad "Transblot" apparatus (Watford, UK). After blotting, the nitrocellulose sheets were labeled with antibodies as described previously (Bradley *et al.* 1988). The determination of protease and periodate sensitivity for JIM13 antigen was as previously described (VandenBosch *et al.* 1989a).

RESULTS

MAC265 antibody reacts with a 95-kD glycoprotein in the lumen of intracellular infection threads in pea nodules (VandenBosch *et al.* 1989a). In the present study, longitudinal sections of whole pea nodules were used to study the expression of this glycoprotein in relation to cell development. Sections of pea nodules were examined in the light microscope after silver-enhanced immunogold labeling. This revealed that the MAC265 antigen in the uninfected nodule parenchyma was confined to apoplastic regions and was concentrated in intercellular spaces (Fig. 2). Labeling could also be seen over infection threads and infection droplets in the central infected region of the nodule, as previously described (VandenBosch *et al.* 1989a).

Examination of thin sections by electron microscopy confirmed that the nodule parenchyma was labeled with MAC265, whereas the adjacent endodermal layer was not labeled. The pattern of labeling in cells of the uninfected nodule parenchyma depended on their stage of develop-

ment. In relatively mature regions of the nodule, uninfected parenchyma cells were separated by large intercellular spaces. While MAC265 gave some labeling at the plasma

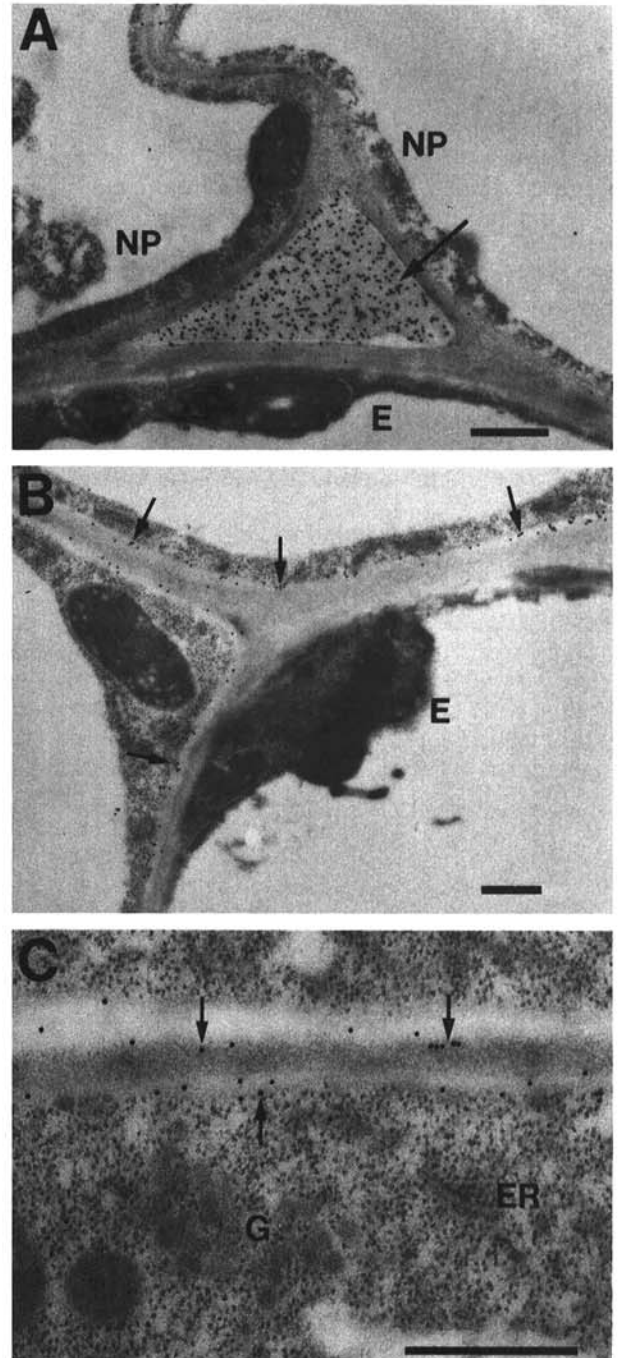


Fig. 3. Electron micrographs of a thin section of a pea nodule after immunogold labeling with MAC265 antibody. Bars represent 0.5 µm. **A**, adjacent to the mature endodermis (E), the MAC265 antigen has accumulated in the intercellular region (arrow) between two cells of the nodule parenchyma (NP). **B**, adjacent to the developing endodermis (E), there are no intercellular spaces, and the MAC265 antigen is visualized at the surface of cells in the nodule parenchyma (arrows). **C**, in cells of the postmeristematic region, which are ahead of the advancing infection threads in the central tissue of the nodule, the MAC265 antigen is seen at the interface between the plasma membrane and the cell wall (arrows). In this section labeling is undetectable over Golgi bodies (G) or ribosomal endoplasmic reticulum (ER).

membrane of these cells, labeling was concentrated in the intercellular spaces (Fig. 3A). The material filling these spaces was distinct from the fibrillar cell walls and had a granular texture but no discernable fine structure. In younger parenchyma tissue underlying the developing endodermis near the apex of the nodule, cells were tightly packed with few intercellular spaces. In these cells, labeling was mainly observed at the interface between the plasma membrane and the cell wall (Fig. 3B) and rarely over the cell wall or in the cytoplasm.

To identify the earliest developmental stage at which nodule cells secrete MAC265 antigen, the apical meristematic region of the nodule was examined. Colloidal gold label associated with MAC265 antigen was observed on the plasma membrane of these uninfected cells (Fig. 3C), which were situated in the region between the meristem and the advancing infection threads. Where the plasma membrane had retracted slightly during fixation, labeling was seen between the membrane and the cell wall. Cytoplasmic organelles, including Golgi bodies and ribosomal endoplasmic reticulum, were not significantly labeled (Fig. 3C).

The evidence presented in Figures 2 and 3 identified three tissue locations for the MAC265 antigen in the pea nodule: the matrix of infection threads and droplets; the surface of uninfected cells at the nodule apex; and the intercellular spaces between the uninfected cells of the nodule parenchyma. To study the developmental links among these three locations, and hence the role of glycoprotein expression in nodule development, other monoclonal antibodies known to react with cell surface glycoproteins were investigated for their pattern of labeling. Six of them react with the 95-kD protein in extracts of pea nodules (Bradley *et al.* 1988, and unpublished results), but when reinvestigated, none of these antibodies was able to discriminate between the three cytological locations for matrix glycoprotein. The survey was then extended to include a group of 10 rat monoclonal antibodies previously selected for their interactions with extracellular glycoproteins that are developmentally regulated in the carrot root apex (Knox *et al.* 1991), because it was hoped that extracellular glycoproteins from carrot might share common epitopes with those from pea nodules (VandenBosch *et al.* 1989a). This possibility was verified by the fact that one of these antibodies that reacted with carrot glycoprotein resembled MAC265 in its immunostaining pattern with pea nodule glycoproteins, both on western blots and on tissue sections (data not shown). Another antibody, JIM13, exclusively labeled a different layer of cells in the uninfected region of the nodule (Fig. 4).

When aqueous extracts of pea nodules were separated by one-dimensional SDS-PAGE and electroblotted, JIM13 showed a pattern of immunolabeling that was distinct from that of MAC265 (Fig. 5). JIM13 reacted with a broad region between 40 and 100 kD, without any individual prominent bands. JIM13 antigen was destroyed by pretreatment of the sample with protease and moreover antibody binding was destroyed by pretreatment of the immunoblot with sodium metaperiodate. These data suggest that JIM13 recognizes a carbohydrate epitope of a glycoprotein.

In a longitudinal section of a pea nodule examined by

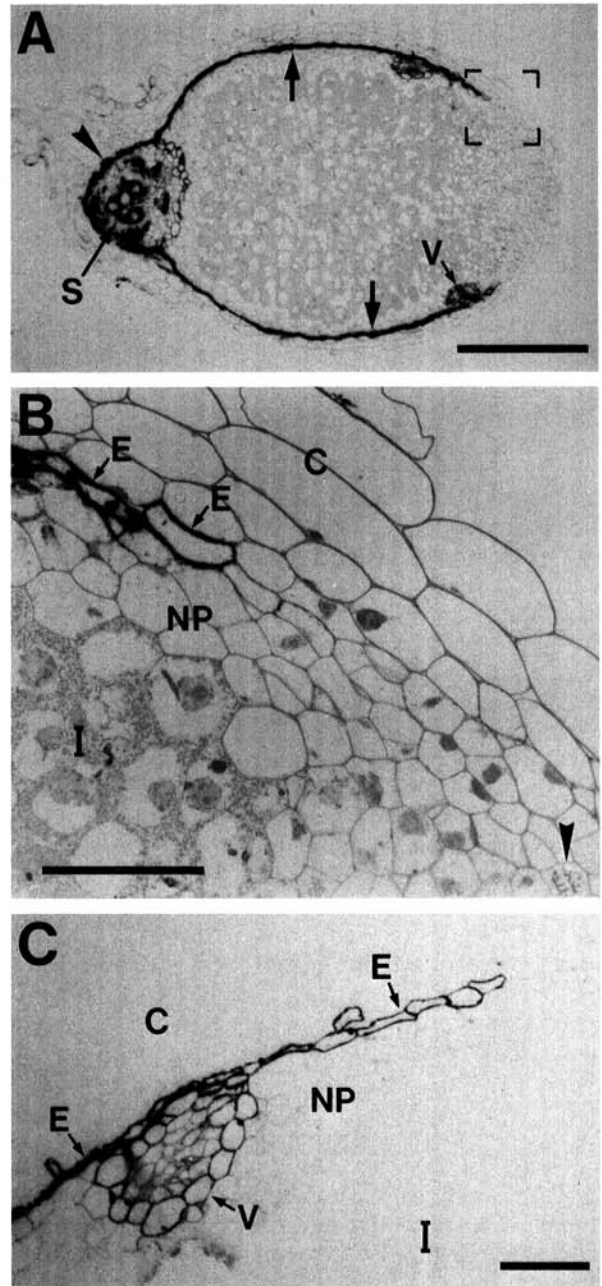


Fig. 4. Light micrographs of a median longitudinal section of a pea nodule after immunogold labeling with JIM13 antibody followed by silver enhancement. Sections A and B have been lightly counterstained with basic fuchsin, whereas section C has not been counterstained. A, black deposits of silver demonstrate the presence of the JIM13 antigen in the nodule endodermis (large arrows) and vascular endodermis (V). In the stele of the root (S), JIM13 labels the endodermis (arrowhead) and metaxylem and also some clusters of cells opposite the protoxylem poles. Bar represents 1.0 mm. B, in an enlarged view of the region indicated by the box corners in (A) the black deposits of silver that correspond to JIM13 antigen are clearly visible against the grey counterstained background. The labeling is confined to the surfaces of cells in the nodule endodermis (E). The outer cortex (C), the nodule parenchyma (NP), and the central infected cells (I) are not labeled by JIM13 antibody. Bar represents 0.1 mm. C, in a similar section without counterstaining only the immunolabeled cells are visible in the nodule endodermis (E) and the vascular endodermis (V). Bar represents 0.1 mm.

light microscopy, JIM13 strongly labeled two arcs of cells in the uninfected cortex (Fig. 4A), continuous with a layer of cells in the root but not extending into the meristematic region at the apex of the nodule. The silver-enhanced gold labeling was restricted to a single layer of cells (Fig. 4B, C), identified as the nodule endodermis by its location and characteristic wall thickenings. The single layer of endodermal cells surrounding each vascular bundle within the nodule parenchyma was similarly labeled (Fig. 4C). In the stele of the root, JIM13 labeled the endodermis and metaxylem and also some small groups of cells situated opposite the protoxylem poles. The labeled cells had extremely thick walls and were possibly phloem fibers (Fig. 4A). In thin sections of nodules examined by electron microscopy, the walls and plasma membranes of nodule endodermal cells were heavily labeled with JIM13 and colloidal gold-linked secondary antibody. In contrast (Fig. 6A,B), JIM13 did not label cells of the uninfected nodule parenchyma nor the nodule outer cortex (the tissue layers that are immediately internal or external to the endodermis). In mature endodermal cells, the cytoplasmic face of the wall was thickened by the addition of an amorphous electron-opaque layer that appeared to exclude or mask the JIM13 antigen (Fig. 6A,B). The peripheral cytoplasm in these cells was reduced in volume and was relatively electron-dense. The JIM13 antigen was present in the cytoplasm and was also concentrated at the plasma membrane on the cytoplasmic face of the wall thickenings.

Using JIM13 antigen as a molecular marker, the pattern of differentiation of the endodermis could be examined in a single median, longitudinal section of a pea nodule, which reveals progressive development from the apical meristem along the longitudinal axis. The most distal cells that showed slight labeling with JIM13 in the light microscope (Fig. 4C) appeared to retain normal cytoplasm and had normal (unthickened) walls (Fig. 6C). In these cells, the antigen recognized by JIM13 was distributed throughout the cell walls and the cytoplasm (Fig. 6C). In even younger cells, JIM13 did not label the cell wall but antigen

was already detectable in cytoplasmic vesicles identified by clusters of gold particles (Fig. 6D). The presumptive vesicles were roughly circular in cross-section and approxi-

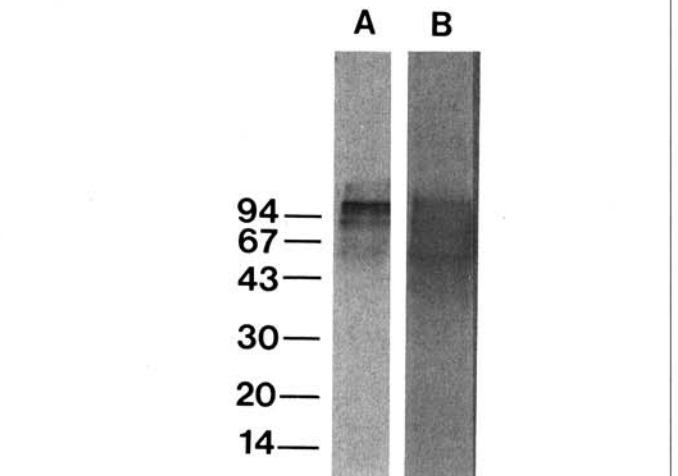


Fig. 5. Identification of antigens in extracts of pea nodules after SDS-PAGE and electroblotting. Immunolabeling was as follows: A, MAC265; B, JIM13. The positions of the molecular weight markers ($M_r \times 10^{-3}$) are indicated at the left.

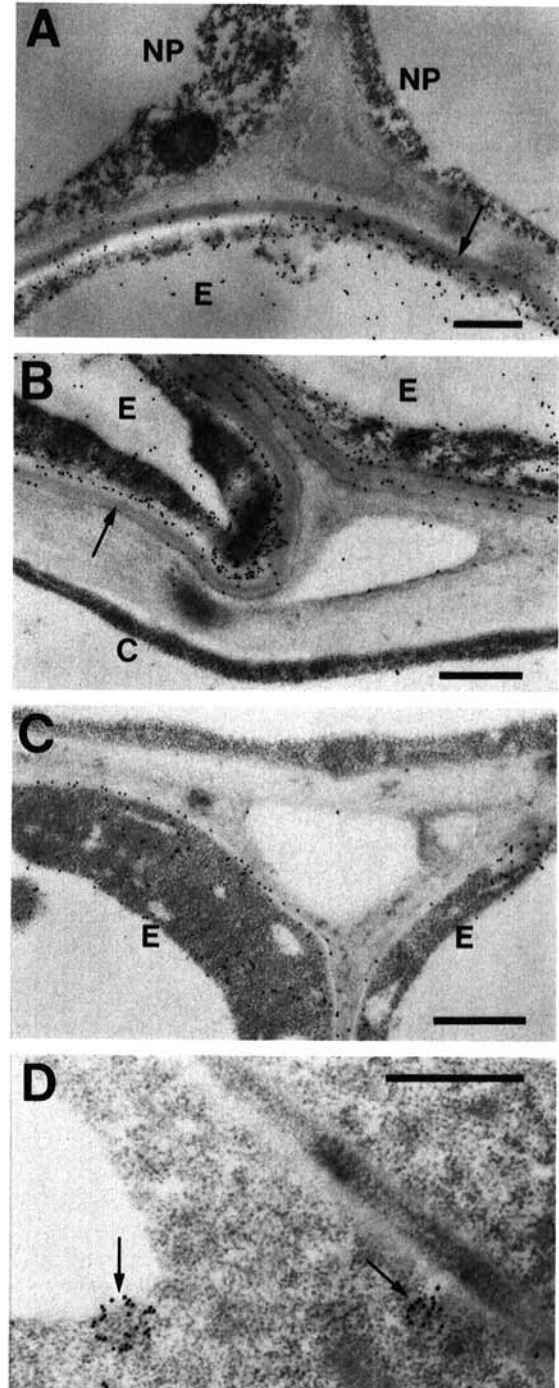


Fig. 6. Electron micrographs of thin sections of pea nodules after immunogold labeling with JIM13 antibody. Bars represent 0.5 μm . A, B, in cells of the mature endodermis (E), the cell wall and plasma membrane are heavily labeled. Note the additional layer in the endodermal cell wall (arrows) that appears to exclude or mask the antigen. There is no labeling in cells of the adjacent layers, the nodule parenchyma (NP) shown in (A), or the nodule cortex (C) shown in (B). C, in cells of the developing endodermis (E) the JIM13 antigen is distributed throughout the cytoplasm and the cell wall, which is not thickened at this stage. D, in undifferentiated postmeristematic cells, JIM13 antibody labels cytoplasmic vesicles (arrows).

mately 200 nm in diameter; however, because osmium was not used as a fixative for these tissues, it was impossible to identify a bounding membrane or internal fine structure. The vesicles were frequently observed close to the plasma membrane (Fig. 6D).

On the roots of some legumes, nodulelike proliferations with peripheral vasculature can be initiated in the absence of tissue invasion by *Rhizobium*. These "empty" or uninvaded nodules have been examined for expression of proteins that are normally associated with nodule development (Van de Wiel *et al.* 1990a). Uninvaded empty nodules can be induced on pea roots by inoculation with strain B659, an LPS-defective mutant of *R. leguminosarum* 3841, which does not appear to induce infection threads on pea or *Vicia*

root hairs after inoculation (E. L. Kannenberg, in preparation). The MAC265 antigen was abundant in the intercellular spaces of the central uninfected tissue of such empty nodules (Fig. 7A), although no bacteria or infection thread structures were visible. On similar tissue sections taken from the same nodule JIM13 labeled two short arcs of cells (Fig. 7B), indicating that differentiation of an endodermal sheath was occurring in the postmeristematic cells of the empty nodule.

DISCUSSION

The distribution of the MAC265 antigen in the uninfected nodule parenchyma (Fig. 2 and VandenBosch *et al.* 1989a) closely parallels that of the early nodulin ENOD2 revealed by *in situ* hybridization (Van de Wiel *et al.* 1990b) with an antisense cDNA probe. The DNA sequence of ENOD2 indicated that this gene encodes a proline- or hydroxyproline-rich cell wall glycoprotein, although no specific antiserum is available to confirm this suggestion by *in situ* localization studies (Govers *et al.* 1990). By contrast, immunogold localization with MAC265 demonstrates that this glycoprotein antigen is localized in the intercellular spaces associated with uninfected nodule parenchyma and also in the central infected tissues of the nodule where it accumulates in the matrix of infection threads. The ENOD2 gene is not transcribed in cells containing infection threads, although a structurally related gene, ENOD12, is expressed here (Scheres *et al.* 1990a). The enhanced expression of MAC265 antigen in the uninfected apical tissues of the nodule (Fig. 3C) as well as in the central infected tissues suggests that its secretion may be a unified plant cell response involved in both *Rhizobium*-induced meristematic activity and *Rhizobium*-induced invasion through infection threads. These morphological responses of the plant to *Rhizobium* infection have previously been considered to be distinct at the molecular level, despite the fact that both are triggered by the same glycolipid signaling molecule produced by the activity of the *Rhizobium nod* genes (Lerouge *et al.* 1990; Truchet *et al.* 1991).

Some aspects of nodule development can take place in the absence of tissue invasion by *Rhizobium* (Van de Wiel *et al.* 1990a). Here, uninvaded pea nodules arising from inoculation with an LPS-defective strain of *R. leguminosarum* were shown to express the MAC265 antigen in intercellular spaces throughout the central tissues. Thus, enhanced secretion of the MAC265 antigen is a very early response to *Rhizobium* infection. This is consistent with data showing that matrix glycoprotein is secreted by root cortical cells in response to a local *Rhizobium* infection (A. L. Rae, in preparation).

In addition to its probable role in tissue and cell invasion by *Rhizobium*, another role for matrix glycoprotein is suggested by its localization in the intercellular spaces of the uninfected nodule parenchyma (Figs. 2, 3). In this location, matrix glycoprotein may contribute to the maintenance of microaerobic conditions in the central part of the nodule, which is essential for nitrogen fixation. Most cells in the nodule are surrounded by a network of gas-filled intercellular spaces where diffusion of oxygen is relatively unimpeded. However, because oxygen diffuses 10^4 times more

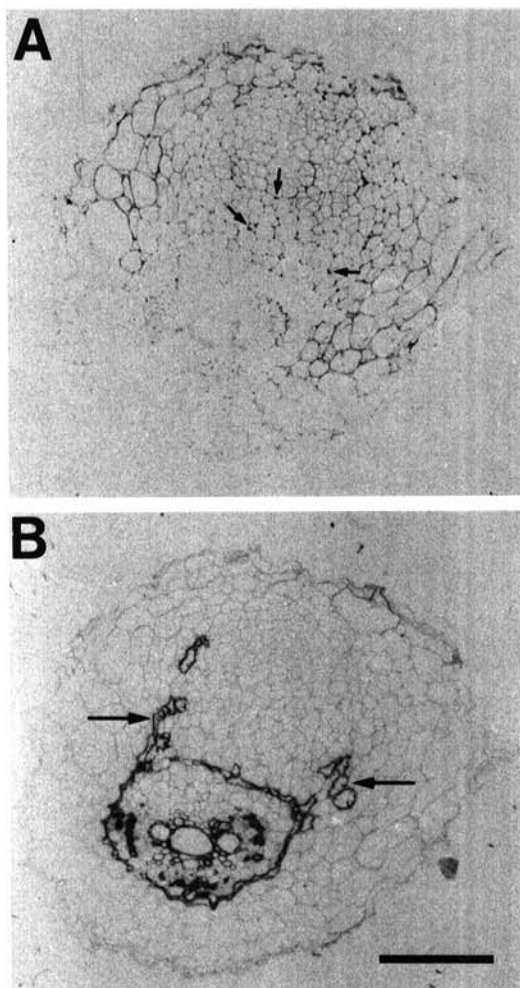


Fig. 7. Light micrographs of longitudinal sections through an "empty" uninvaded nodule developing on a pea root after inoculation with *Rhizobium leguminosarum* strain B659. These sections were very lightly counterstained with basic fuchsin to reveal the complete nodule structure. Bar represents 0.5 mm. **A**, after immunogold-silver staining with MAC265 antibody, labeling is present in the intercellular spaces in the central part of the nodule (arrows). No infection structures or bacteria are visible. The lower half of the section, which corresponds to root tissue, shows very weak labeling. **B**, cells of the incipient endodermis are labeled by JIM13 antibody (arrows). Note that MAC265 and JIM13 show different immunostaining patterns on adjacent tissue sections; thus the immunostaining pattern obtained with MAC265 can be taken as a negative control for JIM13 and *vice versa*.

slowly through water than through air, the major diffusion resistance for oxygen entering the nodule is believed to be a sheath of tightly packed cells without intercellular air spaces (Witty *et al.* 1986). In pea nodules, the most probable site of this barrier to gaseous diffusion is the uninfected nodule parenchyma, together with the tightly packed cells of the apical meristem itself. Intercellular spaces in the uninfected nodule parenchyma are occluded by electron-opaque material and because the glycoprotein that is recognized by MAC265 seems to be a major component of this material, the effective length of the diffusion pathway may be regulated by oxygen-dependent modification of the secretion or water-holding capacity of this glycoprotein. Changes to both the packing of cells and the occlusion of intercellular spaces are seen in soybean nodules grown in abnormal oxygen concentrations (Parsons and Day 1990; James *et al.* 1991).

Monoclonal antibody JIM13 appears to recognize a different class of plant glycoproteins in uninfected pea nodule tissue (Fig. 5). The glycoprotein recognized by this antibody is highly specific to cells of the nodule endodermis, and the developmental sequence of antigen localization suggests that it may have a role in the differentiation of the endodermal cell wall. The extremely specific localization suggests that JIM13 recognizes only one type of glycoprotein, which is polydisperse in molecular weight, because of differing degrees of glycosylation. The antigen is first seen in the cytoplasm of undifferentiated cells (Fig. 6C), and then apparently moves to the cell surface as development proceeds (Fig. 6A,B). Mature cell walls remain heavily impregnated with the JIM13 antigen. In carrot tissue, the epitope recognized by JIM13 belongs to a class of glycoproteins that is developmentally regulated and restricted to certain tissues or cell types (Knox *et al.* 1991). This also appears to be true in pea nodules.

The JIM13 antigen is a useful molecular marker for differentiating endodermal cells in which wall modification is not yet visible. The cytoplasmic expression of JIM13 antigen and the ensuing development of the endodermal sheath are some of the earliest differentiation steps discernable among the uninfected cells derived from the apical meristem of pea nodules. Once the endodermis has been established, its presence might be expected to have a profound influence on the physiological status and hence the developmental fate of neighboring cells and tissues. This suberized cell layer appears to present a barrier to the free diffusion of water, ammonia, and other solutes, and to microbial invasion (Clarkson and Robards 1975; Miao *et al.* 1991). Because both the JIM13 and MAC265 antigens are detectable in the vicinity of the apical meristem, the demarcation between endodermal and parenchymatous cells seems to occur very early in nodule development. The two different cell surface glycoproteins can be used to trace the differentiation of uninfected cell layers in the developing nodule.

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