Molecular composition and development of the plant glycocalyx associated with the peribacteroid membrane of pea root nodules

SILVIA PEROTTO¹, KATHRYN A. VANDENBOSCH¹,³, GEOFFREY W. BUTCHER² and NICHOLAS J. BREWIN¹,*

¹John Innes Institute, John Innes Centre for Plant Science Research, Colney Lane, Norwich, NR4 7UH, UK
²Monoclonal Antibody Centre, AFRC Institute of Animal Physiology and Genetics, Babraham, Cambridge, CB2 4AT, UK
³Present address: Biology Department, Texas A & M University, College Station, Texas 77843-3258, USA
* Author for correspondence

Summary

In root nodules of pea (Pisum sativum), endosymbiotic Rhizobium bacteroids are individually enclosed by a plant-derived membrane, the peribacteroid membrane, creating an organelle-like structure termed the symbiosome. In order to investigate the structure and function of the peribacteroid membrane in plant–microbe surface interactions, monoclonal antibodies were used to identify the major antigenic components of this membrane and to investigate their cellular and tissue distribution. Immunofluorescence studies with isolated intact and ruptured symbiosomes indicated that the relevant antigens were confined to the luminal (inner) face of the peribacteroid membrane. Biochemical analysis indicated that the antibodies recognised carbohydrate epitopes associated with Golgi-derived glycoconjugates of the glycocalyx. Three different classes of glycocalyx antigens, reacting with different groups of monoclonal antibodies, could be distinguished. Immunolocalisation studies of longitudinal sections of pea nodules revealed enhanced expression of glycocalyx antigens in infected nodule tissue, with the three classes of antigen showing different degrees of expression in different developmental zones of the tissue. One of the classes of antigen was absent from the apical meristematic region of pea nodules but was re-expressed during cell invasion by Rhizobium and the development of peribacteroid membranes. In Phaseolus nodules, as opposed to those of Pisum, this group of antigens was entirely lacking from the central infected tissue.

Key words: glycocalyx, peribacteroid membrane, Pisum sativum, plasma membrane, Rhizobium leguminosarum.

Introduction

Rhizobium leguminosarum biovar viciae induces nitrogen-fixing nodules on the roots of Pisum (pea) and Vicia (vetch) seedlings. Bacteria gain access to the cortical tissues of the root through tunnels termed infection threads (Brenchley and Thornton, 1925) which are always bounded by plant cell wall material (Vanden-Bosch et al. 1989a). Subsequently, bacteria leave the infection threads in unwalled infection droplets and are then engulfed by the plant cell plasma membrane (Robertson et al. 1985). After this endocytosis, the bacteria continue to divide and progressively develop into endosymbiotic nitrogen-fixing bacteroids. In pea and clover nodules, the plant-derived peribacteroid membrane partitions in synchrony with divisions of the intracellular bacteria (Robertson et al. 1985), so that bacteroids remain individually enclosed by the peribacteroid membrane, forming organelle-like structures termed symbiosomes (Mellor, 1989; Mellor and Werner, 1987; Roth and Stacey, 1989). It is not known what cell surface interaction drives the internalisation of rhizobia by plant cells, nor what governs the division of peribacteroid membrane envelopes associated with individual bacteria. The most probable mechanism would involve some form of attachment between the peribacteroid membrane and the bacteroid surface, as suggested by observations of electron micrographs (Robertson et al. 1985) and by in vitro studies (Bradley et al. 1986). In order to investigate the molecular basis for surface interactions between the peribacteroid membrane and the endosymbiotic bacteroid, identification of the main components of both the plant and the bacterial membrane is necessary. For this purpose, monoclonal antibodies have proved to be very useful as specific molecular probes (Bradley et al. 1988; Vanden-Bosch et al. 1989a).

The relatedness of the peribacteroid membrane with the plant cell plasma membrane has been emphasised by the recent observation that a monoclonal antibody
first isolated as reacting with peribacteroid membrane (Bradley et al. 1988) recognises a series of plasma membrane glycoproteins that are a common feature of all angiosperms so far tested (Pennell et al. 1989; Pennell and Roberts, 1989). It has been suggested that oligosaccharide side-chains of plasma membrane-bound macromolecules project into the primary plant cell wall (Roberts, 1990), taking the form of a 'glycocalyx' somewhat analogous to the membrane-associated glycoproteins and glycolipids that ensheath animal cells (Alberts et al. 1989). Components of the plant glycocalyx reveal intriguing patterns of developmental regulation that are somehow associated with plant morphogenesis (Knox, 1990). A possible function of the plant cell glycocalyx might be to anchor components of the plant cell wall to the underlying plasma membrane through non-covalent coupling (Roberts, 1990). In this respect, the peribacteroid membrane is exceptional because it is not associated with plant cell wall components such as pectins, xyloglucans and cellulose (VandenBosch et al. 1989a; A.L. Rae, P.Bonfante-Fasolo, unpublished results). The glycocalyx of the peribacteroid membrane may be involved in a similar form of surface interaction, but with the surface carbohydrates of the bacterial outer membrane rather than with the plant cell wall.

Here we describe a biochemical and immunocytological analysis of the major components of the peribacteroid membrane surface that have been identified using monoclonal antibodies as specific molecular probes. We conclude that the peribacteroid membrane carries a differentiated form of glycocalyx composed of a mixture of glycoprotein and glycolipid macromolecules. These glycocalyx antigens are present only on the luminal face of the peribacteroid membrane, where they would be in close contact with the outer membrane of endosymbiotic bacteria. Moreover, an in situ localisation of these membrane antigens on longitudinal sections of pea nodules suggests that they are differentially expressed at successive stages of nodule development.

Materials and methods

Fractionation of nodule and root material

Growth of peas and harvesting of nodules or uninfected roots were as previously described (Bradley et al. 1988). Bean nodules were obtained by the method of Sindhu et al. (1990). The peribacteroid fraction is defined as the material released from membrane-enclosed bacteroids (symbiosomes) by osmotic shock treatment (Brewin et al. 1985): it contains not only peribacteroid membranes, but also the contents of the peribacteroid space. Where necessary, this material was further fractionated by centrifugation at 100,000 g for 30 min in a Beckman TLA 100 bench top ultracentrifuge in order to separate a pellet of peribacteroid membrane from the soluble components of the peribacteroid space. The pellet was then resuspended, sonicated and recentrifuged as described by Norman et al. (1990) to remove contaminants from the soluble material which might have been trapped inside vesicles. A membrane-enriched fraction was derived from uninfected pea roots 14 days after germination. Homogenates were fractionated by centrifugation at 100,000 g for 30 min in a Beckman TLA 100 ultracentrifuge, following a previous low speed centrifugation at 20,000 g for 15 min to remove cell wall debris.

Production of monoclonal antibodies

AFRC MAC 64, 206 and 207 were obtained from previous hybridoma screens (Bradley et al. 1988). Six new fusions were performed, all involving LOU/C rats. Immunisation procedures were all similar to those previously described (Bradley et al. 1988; Galfre' and Milstein, 1981). Myeloma line IR 983F (Bazin, 1982) was used for fusions 1–2, and Y3.Ag1.2.3 (Galfre' et al. 1979) for fusions 3–6. Altogether, approximately 2000 culture supernatants were screened from these six fusion experiments. The selected cell lines were cloned in agar, and subsequently by the limiting dilution method (Galfre' and Milstein, 1981). The classification and grouping of the antibodies selected from the screening were based on a range of biochemical and immunological tests. By these criteria, the antibodies listed here were all distinguishable from each other by epitope specificity. Table 1 lists a representative group of these antibodies. Antibody isotyping was performed using an immunoprecipitation kit provided by Serotec (Oxford, UK). All antibodies were IgM, except MAC 206 and MAC 206 which were IgG3. AFRC MAC 57 is a monoclonal antibody raised in rat and recognizing lipopolysaccharides on the cell surface of R. leguminosarum 3841 (Brewin et al. 1986), the strain that was used for inoculation of pea seedlings. (None of the antibodies used in these experiments and recognising plant membrane antigens cross-reacted with bacterial LPS molecules.)

Immunoblotting

Samples were separated by SDS-PAGE (Laemmli, 1970) using 12% acrylamide mini-gels, with 10 μg protein loaded per lane. The samples were then transferred electrophoretically to membranes of polyvinylidene difluoride (PVDF or Immobilon, Millipore, UK) (Bittner et al. 1980). Immunostaining was visualised using alkaline phosphatase conjugates as previously detailed (Bradley et al. 1988).

Treatment with sodium metaperiodate

To test for sensitivity to periodate oxidation (Woodward et al. 1985), peribacteroid material (0.5 μg protein) was immobilised on nitrocellulose sheets (Schleicher and Schuell, Dassel, Germany) as a series of dots for immunostaining. The sheets were dried and then equilibrated with sodium acetate buffer (50 mM, pH 4.5), followed by incubation in the dark for 1 h at 25°C in the same buffer containing sodium metaperiodate at 0, 20, 100 or 200 mM. The sheets were rinsed and then incubated for 30 min, with either 50 mM sodium borohydride or 1% (w/v) glycine in Tris-buffered saline (TBS 50 mM Tris–HCl, 150 mM NaCl, pH 7.5).

Treatment with proteinase K

Samples of peribacteroid membranes for protease treatment were prepared without the normal inclusion of a protease inhibitor (p-aminobenzamidine). This fraction was diluted to a final antigen concentration of 1 mg ml\(^{-1}\) protein, mixed with an equal volume of Laemmli solubilisation buffer and treated with proteinase K as described by Sindhu et al. (1990). Aliquots equivalent to 10 μg protein for the undigested antigens were fractionated by SDS–PAGE (together with the untreated controls), electroblotted to nitrocellulose or PVDF sheets, and immunostained.

Sensitivity to detergents

Nitrocellulose sheets carrying antigen dots (0.5 μg protein)
were soaked in TBS and incubated for 30 min with either polyoxyethylene sorbitan monolaurate (0.5% v/v Tween 20 in TBS) or sodium dodecyl sulphate (0.1% w/v SDS in TBS). After five rinses in TBS to remove detergent, the nitrocellulose sheets were incubated with 2% bovine serum albumin (BSA) and immunostained in the normal way.

**Lipid extractions**

Lipids were extracted by the method of Folch et al. (1957). The membrane material was dissolved in water and 20 vol of a chloroform/methanol (2:1 vol/vol) solution was added. After standing for 15 min, the sample was centrifuged at 1500 g and 1/5 vol of 50 mM CaCl₂ was added to the supernatant. The two phases were then separated by centrifugation at 1500 g and the water/methanol phase was dialysed against deionised water. Both fractions were reduced in volume, applied as dots onto membrane and immunostained with antibodies.

**ELISA competitive binding assays**

To test for competitive inhibition by sugars, 96-well microtitre plates were coated with antigen by incubation for 16 h at 4°C with 50 µl peribacteroid material (3 µg ml⁻¹ protein in TBS containing 0.01% sodium azide). Competition of binding was performed following the protocol described by Knox et al. (1989). Each potentially competing sugar was used at a final concentration of 100 mM from a freshly prepared 1 M stock solution in TBS adjusted to pH 7.5. At least five replicates were made for each sugar treatment.

**Purification of intact peribacteroid units**

Intact membrane-enclosed bacteroids (symbiosomes) were purified from pea nodules by separation on a step gradient of Percoll (Pharmacia, Sweden) containing 350 mM mannitol, as described by Price et al. (1987). Symbiosomes were recovered as a single band between 30% and 60% Percoll and resuspended in washing buffer (25 mM Tris–HCl, 350 mM mannitol, 3 mM MgSO₄, pH 7.5) containing 1% BSA. Symbiosomes were allowed to attach to the surface of poly-L-lysine-coated glass microscope slides overnight at 4°C in a moist chamber. To rupture the peribacteroid membrane, slides were immersed for 10 min in 25 mM Tris–HCl, 3 mM MgSO₄, pH 7.5. After osmotic shock, the samples were overlaid with 1% BSA in washing buffer for 1 h before immunostaining.

**Microscopy**

Nodule tissue was embedded in LR white resin and used for immunogold staining and silver enhancement as previously described (VandenBosch et al. 1989b). To achieve satisfactory fixation of the central tissues, pea nodules were pierced with a pin during fixation. These punctures are visible on the lower side of the sections in Fig. 8A, C.

**Results**

**Identification of membrane components using monoclonal antibodies**

To resolve membrane and soluble components, the peribacteroid material obtained by osmotic shock treatment of intact symbiosome units was fractionated into peribacteroid membrane (pbm) and peribacteroid soluble (pbs) components by ultracentrifugation. Following SDS–PAGE and electroblotting to PVDF (Immobilon) membranes, the immunostaining patterns for all the antibodies listed in Table 1 fell into four classes (Fig. 1). Class I antibodies, typified by MAC 207, bound to a range of bands (Mr 45-120×10³) derived from the pbs sample. Class II antibodies (MAC 206, 255 and 268) reacted mainly with comple-
Monoclonal antibodies reacting with antigens on the peribacteroid membrane

<table>
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<th>Antibody</th>
<th>Periodate sensitivity of epitope</th>
<th>Nature of antigen</th>
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Antibodies are divided into Classes I-IV on the basis of antigen binding on immunoblots (Fig. 1).

Characterisation of carbohydrate epitopes

As shown in Table 1, antibody binding was often sensitive to metaperiodate oxidation of the antigen, indicating that these epitopes had a carbohydrate component. The nature of these epitopes was further investigated by using various sugars as competitive inhibitors of the antibody–antigen binding reaction in an ELISA assay system (Table 2). L-arabinose and D-melibiose (6-0-alpha-D-galactopyranosyl-D-glucose) were the most commonly effective competitors for binding of antibodies of classes I, II and III. However, the antigen recognised by MAC 275 was unique in providing no evidence of being a glycoconjugate. The sensitivity of MAC 275 antigen to protease digestion (data not shown) and the clearly defined band identified on immunoblots (Fig. 1) suggest that a single polypeptide was involved in this case. (Further analysis of this antigen will be presented elsewhere.)

Intracellular localisation of antigens

Localization of MAC 255 antigen around the endosymbiotic bacteroids was demonstrated by silver enhancement after immunogold staining of infected cells in nodule tissue sections (Fig. 2). A similar localisation was also observed for all the other antibodies recognising glycoconjugates (Table 1, classes I–III). These results were confirmed by electron microscopy following immunogold staining of sections of nodule tissue (data not shown). In addition to labelling peribacteroid and plasma membranes, class III antibodies (MAC 254...
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Fig. 3. Electron micrograph showing presence of MAC 254 antigens in Golgi body (g) and peribacteroid membrane (p) by immunogold staining of nodule thin sections using 15 nm gold anti-rat IgG. Bacteroids (b) are unlabelled. Bar=200 nm.

and MAC 266) gave exceptionally strong labelling of Golgi bodies (Fig. 3).

Localisation of antigens to the luminal face of the peribacteroid membrane

Evidence that the antigens recognised by these monoclonal antibodies are present only at the luminal (inner) surface of the pbm was obtained by immunofluorescence studies with intact or ruptured symbiosomes isolated from pea nodules in the presence of 350 mM mannitol as osmotic protectant (Fig. 4). When a preparation of membrane-intact symbiosomes was examined, only a minority (<10%) of the bacteroid units stained with MAC 255, MAC 266 or any of the antibodies listed in Table 1, showing that the corresponding antigens were not present on the cytoplasmic face of the pbm. In a control experiment, a similar frequency of immunostaining (<10%) was observed with MAC 57, an antibody that recognises lipopolysaccharides (LPS) on the bacterial surface (Brewin et al. 1986). This experiment served as an indicator of the frequency of symbiosomes with ruptured pbm, because the LPS antigen would only have been exposed when the peribacteroid membrane had already been perforated before labelling with the antibody. In order to rupture the pbm, the preparation of membrane-intact symbiosomes was shocked by transfer to a buffer lacking osmotic protectant. When immunostained after this treatment, all bacteroids were strongly and uniformly stained with MAC 57 anti-bacterial LPS antibody. In all of these ruptured samples, the monoclonal antibodies reacting with pbm also gave good immunofluorescence staining of bacteroid-associated material. In particular, 'ghost' fragments of pbm could be visualised which had been attached to the poly-L-lysine-coated slides before release from bacteroids by osmotic shock treatment. These results demonstrate that the glycoconjugate antigens recognised by the antibodies listed in Table 1 were localised predomi-

Fig. 4. Immunofluorescence localisation of bacterial lipopolysaccharide and peribacteroid membrane antigens in isolated symbiosomes. Preparations of symbiosomes were either immunolabelled directly (a) or after osmotic shock treatment (b) to rupture the peribacteroid membrane and to reveal antigens present in the lumen of the symbiosomes. Each block shows fluorescent immunolabelling (lower micrograph) and the corresponding phase-contrast image (upper micrograph) for MAC 57 (anti-bacterial LPS); MAC 255 (anti-pbm); MAC 266 (anti-pbm). 'Ghost' fragments of pbm after release of bacteroids are visible (white arrow).
Fig. 5. Immunoblots of peribacteroid membrane samples fractionated on SDS-PAGE after protease pretreatment (+) and electroblotted onto PVDF membrane using MAC 207 (class I), MAC 268 (class II) and MAC 254 (class III). The control lanes (−) represents undigested samples.

nantly or exclusively on the luminal surface of the peribacteroid membrane.

Biochemical nature of glycoconjugate macromolecules
To gain further information on the nature of these glycoconjugates, samples were digested with protease, separated by SDS-PAGE and immunostained after transfer to blotting membranes (Fig. 5). Analysis of immunoblots revealed that the high molecular weight antigen bands recognised by class I and class II antibodies (exemplified by MAC 207 and MAC 268 respectively) were protease-sensitive, demonstrating that the corresponding carbohydrate epitopes were parts of glycoproteins (Fig. 5). Class I and II antibodies also reacted with high molecular weight protease-insensitive components that appeared as faint smears on nitrocellulose membranes (data not shown) but were more evident when hydrophobic blotting membranes such as PVDF were used (Fig. 5). All class II antibodies bound strongly to fast-migrating components of the pbm that were protease-insensitive (Fig. 5), whereas all the antigen bands recognised by class III antibodies (e.g. MAC 254) were protease-sensitive.

To investigate whether the protease-insensitive low molecular weight antigens recognised by class II antibodies might be glycolipids, peribacteroid components were partitioned between aqueous methanol and chloroform phases and the fractions analysed by dot immunoassay (Fig. 6). Only antibodies of class II recognised antigens that partitioned into the chloroform phase. These chloroform extracts were fractionated by SDS-PAGE, and after electroblotting it was confirmed that the samples contained the low M_r antigens recognised by MAC 206 and other class II antibodies (data not shown).

Because the fast-migrating antigens recognised by class II antibodies are protease-insensitive and extractable in organic solvents, it is probable that they are plant membrane glycolipids. It was also noted that class II antibodies failed to react with immunoblots if the washing buffer included Tween 20 (0.5 % w/v), perhaps indicating that these fast-migrating antigens were attached to blotting membranes through hydrophobic rather than electrostatic interactions.

Comparison of peribacteroid membranes with membranes from uninfected roots
To investigate the developmental regulation of the expression of membrane antigens, membrane samples from uninfected pea roots were compared with peribacteroid membranes after immunoblotting (Fig. 7). The size distribution of antigens recognised by class II antibodies (e.g. MAC 268) was significantly different on the peribacteroid membrane preparation relative to the membrane preparation from uninfected roots. Some of the glycoprotein antigens recognised by class I anti-

Fig. 6. Dot immunoassays showing partitioning of peribacteroid membrane antigens into aqueous methanol or chloroform phases. (A) starting peribacteroid membrane material; (B) methanol/chloroform-insoluble fraction; (C) aqueous methanol soluble fraction; (D) chloroform-soluble fraction. MAC 207 represented all class I antibodies (Table 1); MAC 254 represented class III antibodies; MAC 268 represented all class II antibodies except MAC 255 (illustrated) which showed a slightly different antigen distribution between aqueous methanol and chloroform phases.

Fig. 7. Immunoblots of uninfected pea root membranes (r) and peribacteroid membranes (p) stained with MAC 207 (class I), MAC 268 (class II), MAC 254 (class III).
bodies (e.g. MAC 207) seemed to be less abundant in the pbm fraction relative to the root membrane fraction.

Tissue distribution of antigens in nodule sections

In order to investigate the tissue distribution of glycocalyx components, semi-thin longitudinal sections of pea nodules were incubated with monoclonal antibodies and observed after immunogold staining and silver enhancement (Figs 8 and 9). As a control experiment, MAC 57 (an anti-bacterial LPS antibody) was used to demonstrate the presence of bacteria in infected nodule tissue and the absence of non-specific staining on uninfected plant tissue (Fig. 8B). MAC 207 (class I) showed very intense labelling of the meristematic region and the early infected tissue of the nodule, while the intensity of labelling decreased in the infected cells of the mature symbiotic zone (Fig. 9A,D). Labelling was also present on all other non-infected parts of the nodule and on root tissues.

MAC 255 typified the labelling pattern given by antibodies of class II (Fig. 9B). In contrast to the pattern shown for MAC 207, labelling with MAC 255 was almost negative in the region of the nodule meristem (Fig. 9E). The detectability of MAC 255 antigen increased dramatically at the point in nodule development where endocytosis of symbiotic bacteria takes place and became very strong on membranes in infected regions of the nodule. On nodule sections, MAC 255 also labelled the plasma membrane of cells of the outer cortex and of nodule parenchyma, as well as cells of the root cortex and root epidermis. However, tissues from the root central cylinder and the nodule vascular bundles were negative after labelling with MAC 255.

Labelling with MAC 266 (class III) was detected in all tissues, but the intensity of immunostaining increased in infected cells from older parts of the nodule (Fig. 9C,F).

To compare the distribution of the corresponding antigens in a determinate nodule, sections of young Phaseolus nodules were examined with antibodies that reacted with each of the three groups of glycocalyx components from pea nodules (Fig. 10). While class I antigens were strongly represented in the membranes of the infected Phaseolus cells (Fig. 10A), class II antigens were completely absent from the central tissues of the developing nodules (Fig. 10B). Class III antigens were uniformly weak in all tissues of developing Phaseolus nodules (data not shown).

Discussion

During the early stages of nodule development, extracellular plant glycoproteins appear to be involved in many aspects of plant cell differentiation and invasion with Rhizobium. Previous reports have identified a glycoprotein component of the infection thread lumen and intercellular space (VandenBosch et al. 1989a); a proline-rich cell wall glycoprotein associated with the differentiation of uninfected nodule parenchyma (van de Wiel et al. 1990); and some genes encoding other extracellular glycoproteins whose distribution is more closely correlated with cell invasion by Rhizobium (Scheres et al. 1990a,b). The present study extends this analysis to the peribacteroid membrane surface, which is shown to be a developmentally regulated form of the plasma membrane surface.

The monoclonal antibodies described in this study (classes I–III, Table 1) identify a range of epitopes associated with glycoprotein and glycolipid components of the peribacteroid membrane (Figs 1–3). It was demonstrated by immunofluorescence (Fig. 4) that the antigens were not exposed on the cytoplasmic surface of the peribacteroid membrane but occurred on the luminal (inner) face, contiguous with the surface of the endosymbiotic bacteroids. This face is topologically equivalent to the external face of the plasma membrane, and the carbohydrate structures that it carries can be collectively considered as a glycocalyx (Roberts,
It has recently been shown that the glycocalyx of animal cells is commonly involved in physical interactions with the surface of pathogenic bacteria (Karlsson, 1989), and that glycolipid components are particularly important in this respect. Similarly, some of the glycocalyx antigens associated with the peribacteroid membrane may have an important role in the plant–microbial surface interaction that leads to endocytosis and the development of symbiosomes (Roth and Stacey, 1989).

As a result of the endocytosis of *Rhizobium* and the consequent development of symbiosomes, there is an enhanced synthesis of peribacteroid membrane material (Robertson *et al.* 1985) so that, in mature infected cells, the surface area of peribacteroid membrane is probably 50–100 times that of the plasma membrane (Mellor and Werner, 1987). This increase in the total membrane content of infected cells is reflected in an increased total immunostaining of glycocalyx antigens in these cells relative to cells of uninfected tissue.
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Fig. 10. Transverse section of *Phaseolus* nodules showing immunogold staining and silver enhancement following treatment with (A) MAC 207 (class I) and (B) MAC 255 (class II). The cortical tissues (c) are clearly distinguishable from the central infected region (i) of the nodule. Bar=100 μm.

(Figs 8, 9). However, superimposed on this general increase in the abundance of glycocalyx antigens, it was possible to observe differences in the expression of particular antigens during the development of symbiosomes, as revealed by *in situ* studies on longitudinal sections of pea nodules (Figs 8, 9). Glycoproteins recognised by class I antibodies (e.g. MAC 207) appeared to be strongly represented on the membranes of the infection and early symbiotic zone but diminished in the mature symbiotic zone. These antigens were also expressed on the membrane of uninfected cells of the nodule and in the root (Fig. 9A). Indeed, antigens recognised by MAC 207 have been reported to be a common feature on the plasma membrane of angiosperms (Pennell *et al.* 1989). They have been biochemically characterised as plasma membrane-associated arabinogalactan proteins (AGPs). Recently a nodule-specific gene transcript, ENOD5, has been identified in pea nodules, which may encode an arabinogalactan protein (Scheres *et al.* 1990b). Expression of the ENOD5 gene is particularly enhanced in the region of the nodule corresponding to the early symbiotic phase, which correlates with the stage of bacterial release and development of the peribacteroid membrane. Since this is also the zone where MAC 207 antigen shows enhanced expression, it is possible that the polypeptide product of the ENOD5 gene is decorated with the oligosaccharide antigen recognised by MAC 207 or a related class I antibody. AGPs have been shown to be developmentally regulated (Fincher *et al.* 1983; Knox, 1990), and the different topological and temporal expression of particular epitopes may perhaps be involved in modulating cell–cell surface interactions. Similarly, their enhanced expression in the early stages of infection may indicate a role in surface interactions between the plant membrane and the bacterial surface.

In contrast to the antigens recognised by class I antibodies, the expression of components recognised by MAC 255 and all other class II antibodies remained strong and uniform on the glycocalyx of the peribacteroid membrane, starting from the region of endocytosis and stretching right across the zone of infected plant cells in the central tissues of the nodule. A biochemical analysis shows that the arabinose-containing epitopes recognised by the antibodies of class II are common to glycolipids and glycoproteins. Previous reports also indicate that similar glycosidic components rich in arabinose and galactose can be carried on both glycolipids and glycoproteins in pea membranes (Hayashi and Maclachlan, 1984; Pillonel and Maclachlan, 1985). The glycolipids recognised by class II antibodies may serve either as a precursor or as an alternative acceptor for the arabinogalactan groups that are targeted to membrane AGPs. However, the relationship between these two groups of membrane antigens remains to be elucidated.

Remarkably, the meristematic cells which gave rise to infected tissue in pea nodules showed very little expression of MAC 255 antigen (Fig. 9B,E). Because this antigen was present in the cortical cells of the root that gave rise to the nodule meristem (Fig. 9B), this observation implies that the MAC 255 antigen was first lost during dedifferentiation of root cortical cells into the nodule meristem and then regained at the time of invasion by bacteria. However, in *Phaseolus* nodules, the antigens recognised by all class II antibodies were entirely lacking from the central infected zone of the nodule (Fig. 10B). It is interesting to note that, during the development of determinate nodules such as *Phaseolus* (bean), cell invasion by *Rhizobium* occurs in root cortical cells and precedes the induction of meristematic activity (Rolfe and Gresshoff, 1988). Thus the infected cells of the central region of bean nodules are themselves meristematic, in contrast to the situation in pea nodules where the apical meristem is uninfected and only postmeristematic cells become invaded by *Rhizobium*. This difference in the sequence of events during the development of determinate (bean) and indeterminate (pea) nodules correlates in an interesting way with differences in the expression of class II glycocalyx components in infected cells (Figs 9B and 10B).
The third class of antibodies (e.g. MAC 266) recognised antigens that showed enhanced expression in the infected regions of the pea nodule that are associated with increasing maturity and senescence. This class of antibodies recognises an epitope carried by membrane antigens and by soluble components of the peribacteroid fluid (Fig. 1). However, the relationship between membrane and soluble glycoproteins has yet to be established. Some of these soluble glycoprotein components may correspond to acid hydrolases (M.F. LeGal, personal communication), and it is possible that the increase in labelling on longitudinal sections of pea nodules (Fig. 9C) may correspond to the accumulation of lytic enzymes in the peribacteroid space during senescence.

In summary, these results demonstrate that the peribacteroid membrane glyocalyx carries developmentally regulated antigens that increase or decrease in abundance according to the stage of nodule development. The expression of these antigens in the peribacteroid membrane may be crucial to the development and maintenance of the symbiotic association between plant cells and bacteroids.

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