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Common components of the infection thread matrix and the intercellular space identified by immunocytochemical analysis of pea nodules and uninfected roots

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Three rat hybridoma cell lines have been isolated which produce monoclonal antibodies identifying a nodule-enhanced, soluble component of *Pisum sativum* root nodules. These antibodies each recognized a protease-sensitive band (M_r 95K) on SDS-polyacrylamide gels. The 95K antigen was resolved by isoelectric focusing into acidic and neutral components which were separately detected by AFRC MAC 236 and MAC 265 respectively. The third antibody (MAC 204) reacted with both acidic and neutral components through an epitope that was sensitive to periodate oxidation. These monoclonal antibodies were used for immunogold localizations at light and electron microscopic levels. In each case, the antigen was shown to be present in the matrix that surrounds the invading rhizobia in infection threads and infection droplets, as well as in the intercellular spaces between plant cell walls of nodules and also of uninfected roots. By contrast, a fourth monoclonal antibody, AFRC JIM 5, labelled a pectic component in the walls of infection threads, and JIM 5 was also found to label the middle lamella of plant cell walls, especially at three-way junctions between cells. The composition and structure of the infection thread lumen is thus comparable to that of an intercellular space.

Key words: infection thread/nodules/*Pisum sativum*

Introduction

The infection thread is an intracellular tunnel through which invading rhizobia are conducted across the legume epidermal root hair cell and also across the cortical cells of the developing root nodule (Turgeon and Bauer, 1985). This tunnel is sheathed by plant cell membrane and plant cell wall materials (Callaham and Torrey, 1981). Rhizobia do not usually develop the capacity to fix nitrogen within the infection thread (de Faria *et al.*, 1986), which represents only the first stage in the process of internalization of bacteria by plant cells. Subsequently the rhizobia penetrate the plant cell wall (Mort and Grover, 1988), and are released into an 'infection droplet' structure contained by an invagination of the plant cell membrane (Robertson *et al.*, 1985). Individual bacterial cells are then internalized into the plant cell cytoplasm, where they normally develop into nitrogen-fixing endosymbiotic bacteroids enclosed by a plant-derived peribacteroid membrane (Brewin *et al.*, 1985). However, not all legumes are invaded by rhizobia through the development of infection threads (Rolfe and Gresshoff, 1988; Torrey, 1988). The alternative route involves 'crack entry' through the root epidermis and/or the root cortex by way of intercellular spaces or the middle lamella as, for example, in *Arachis* (Chandler, 1978) and *Stylosanthes* (Chandler *et al.*, 1982). Our present data reconcile these two apparently different modes of entry by suggesting that the infection thread should be considered as a specialized anatomical modification of the normal intercellular space, rather than as a unique symbiosis-specific structure.

We have used monoclonal antibodies to identify a 95K plant component which was considerably more abundant in developing pea nodules than in uninfected root tissue. Information concerning the cytological distribution of this antigen was obtained by immunogold labelling, which showed it to be a major component of the infection thread matrix. Additionally, silver enhancement of immunogold labelling permitted investigation by light microscopy, and this revealed an extracellular location for the 95K antigen in intercellular spaces. Three antibodies reacting against infection thread matrix antigens, and a fourth that reacts with cell wall pectin, were used to re-examine the relationship between the infection thread and the intercellular spaces formed at the junctions between plant cell walls during the normal process of cell division (Jeffree *et al.*, 1986).

Results

Screening of antibodies by Western blot analysis

Root and nodule proteins were examined after one-dimensional SDS-PAGE, electroblotting and immuno-staining with the monoclonal antibodies MAC 204, MAC 236 and MAC 265 (Table I). The resulting blots are pictured in Figure 1. All three antibodies recognized a prominent, diffuse band in nodule tissue, ~95 kd in mol. wt. MAC 204 and MAC 265 also recognized a similar band in young root tissue, although reaction with root tissue was rather faint when MAC 236 was used as a probe. Incubation of nitrocellulose blots with MAC 83 (an irrelevant antibody) resulted in no non-specific labelling of plant components.

After two-dimensional electrophoresis of the nodule supernatant fraction, the 95K band was resolved into a strongly acidic form and a neutral (or slightly alkaline) form of the antigen (Figure 2), and these two forms showed different antibody specificities. MAC 204 recognized both forms of the macromolecule, whereas MAC 236 detected

Table I. Monoclonal antibodies

Name	Antigens recognized	Isotype	Reference
AFRC MAC 204	Matrix glycoprotein of infection threads	IgM	Bradley *et al.*, 1988
AFRC MAC 236	Matrix glycoprotein of infection threads	IgG_{2b}	This study
AFRC MAC 265	Matrix glycoprotein of infection threads	IgG_{2a}	This study
JIM 5	Polygalacturonic acid	IgG_{2a}	This study

only the acidic form and MAC 265 detected only the neutral form. An immunoaffinity column was constructed by coupling MAC 236 to sepharose, and this was used to isolate MAC 236 antigen from the nodule supernatant fraction. After elution of the material retained on the column, this was subjected to gel electrophoresis and immunoblotting. As expected, a 95K band could be detected by immunostaining with MAC 236, but this band was also found to cross-react with MAC 204, suggesting that both epitopes are carried on the same macromolecule.

Biochemical analysis of the antigens

Pre-treatment of the nodule supernatant fraction with proteinase K prior to electrophoresis and electroblotting resulted in the complete removal of all three antigens from immunoblots stained with MAC 204, 236 and 265, indicating a protein component for these antigens. Similarly, pretreatment of immunoblots with 200 mM sodium metaperiodate under acidic conditions resulted in complete loss of the ability to bind MAC 204 antibody, indicating a carbohydrate epitope for both acidic and neutral components. The epitopes recognized by MAC 236 and MAC 265 were resistant even to high concentrations of periodate (200 mM), but because these epitopes are carried on the same macromolecules that carry the MAC 204 epitope, it is concluded that all three antibodies recognize related plant glycoproteins. Further evidence that the antibodies recognized carbohydrate groups was sought by monitoring antigen–antibody interactions in ELISA assays under conditions where the addition of monosaccharides or complex glycoproteins might interfere with the binding reaction (Anderson *et al.*, 1987). It was shown that Gum arabic (50 μg/ml), a complex extracellular glycoprotein from *Acacia senegal* (Fincher *et al.*, 1983), gave >90% inhibition of antigen binding for MAC 204 and ~50% inhibition for MAC 236 and MAC 265. However, none of the following sugars (100 mM) were inhibitory for antigen–antibody binding in an ELISA competition assay: L-arabinose, D-galactose, D-glucuronic acid, D-galacturonic acid, L-rhamnose, D-arabinose, D-glucose, D-mannose, D-xylose, L-fucose and *N*-acetyl D-glucosamine. These negative data might be consistent with epitopes that were complex carbohydrates, rather than simple repeating sugar homopolymers, but a more detailed characterization of the epitopes recognized by MAC 204, 236 and 265 must await purification of the antigens in larger quantities than have been obtained so far.

Immunocytochemical labelling of infection thread matrix and wall components

Following immunogold labelling and silver enhancement (Figure 3), the distribution of antigens detected by MAC 204, 236 and 265 was found to be identical. Heavy labelling was observed over infection threads and unwalled infection droplets. Additionally, label occurred over some intercellular

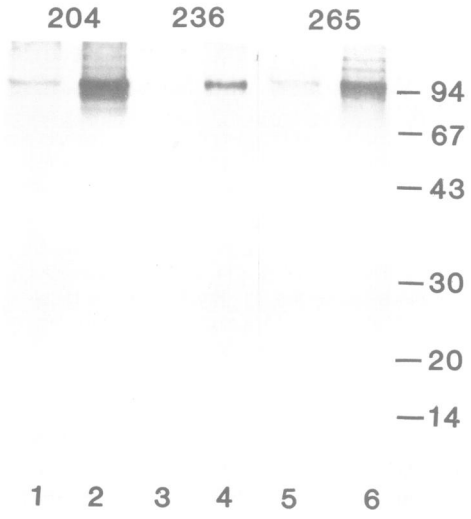

Fig. 1. Immunochemical identification of infection thread glycoproteins using monoclonal antibodies MAC 204, MAC 236 and MAC 265, after one-dimensional gel electrophoresis, electroblotting to nitrocellulose sheets and treatment with anti-rat IgG antibody conjugated to alkaline phosphatase. **Lanes 1, 3** and **5** contain root proteins and **lanes 2, 4** and **6** contain nodule supernatant proteins. Positions of the mol. wt markers (Pharmacia, UK) are indicated at the right.

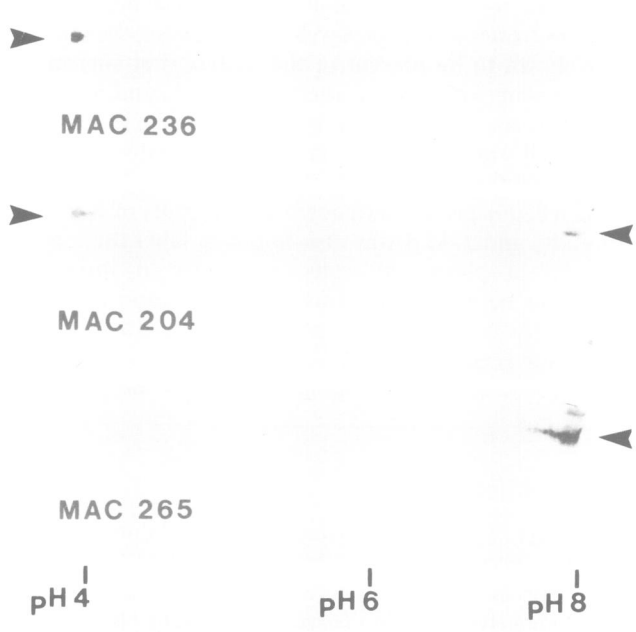

Fig. 2. Immunochemical staining of infection thread glycoproteins from pea nodules after two-dimensional gel electrophoresis, electroblotting and immunostaining with MAC 236, MAC 204 and MAC 265. High mol. wt regions of each gel are shown and the position of the 95K antigen is indicated by arrows. The pH values were deduced by slicing a tube gel into 0.5 cm segments and equilibrating each slice with 0.5 ml 'Analar' water for 30 min.

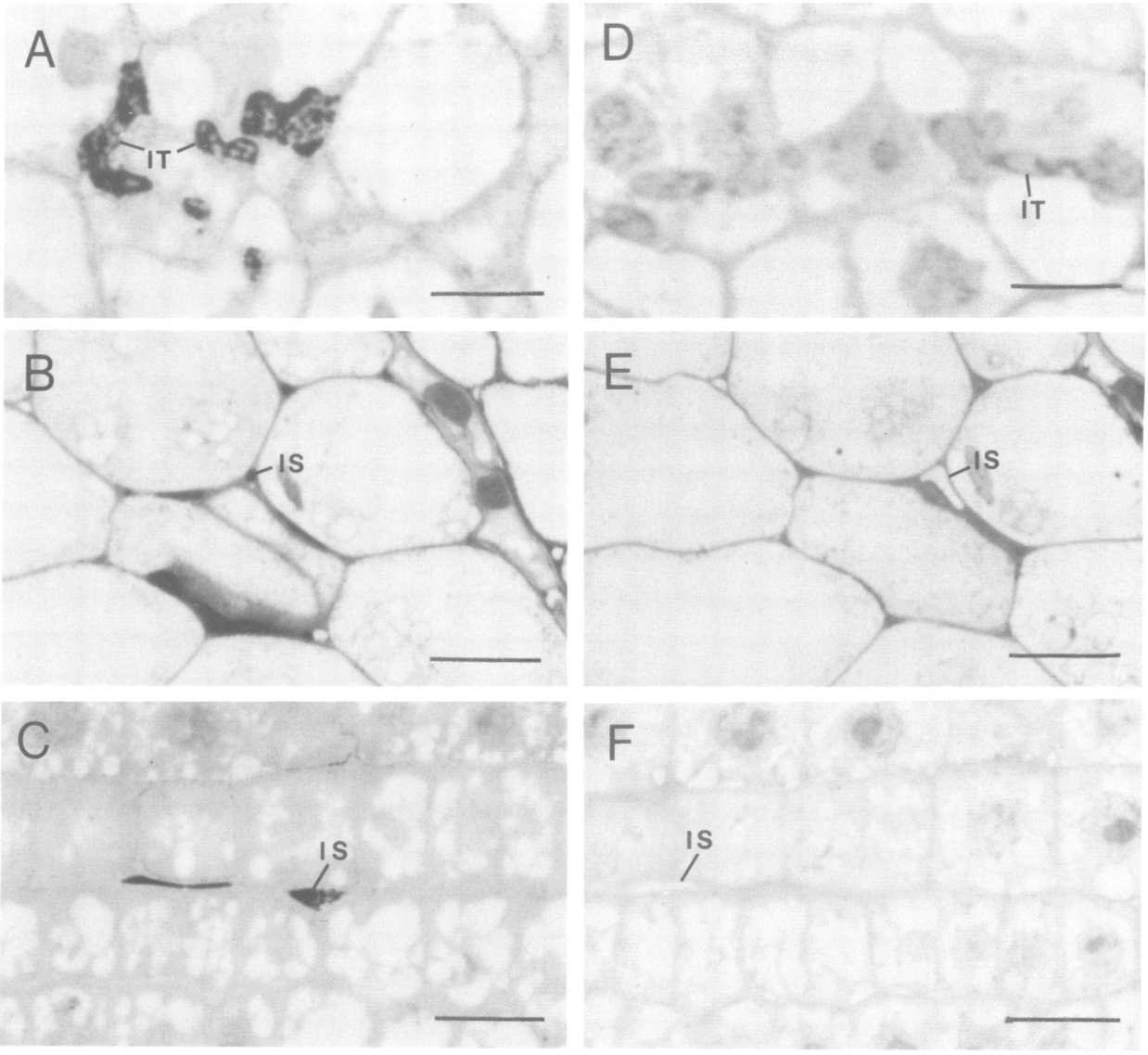

Fig. 3. Localization of the MAC 236 antigen following immunogold labelling and silver enhancement. **Views A, B** and **C** depict labelling after incubation with MAC 236. Omission of the primary antibody results in no labelling of comparable adjacent sections (**D, E, F**). Bars, 20 μm. 800 ×. (**A**) Labelling of young, infected tissue in a pea nodule. Dense silver deposits indicate the presence of the MAC 236 antigen in infection threads (IT). (**B**) Some intercellular spaces (IS) in the uninfected nodule cortex clearly contain the MAC 236 antigen. (**C**) MAC 236 identifies a component of intercellular spaces in cortical tissue from uninfected pea roots.

spaces, notably in the inner cortex of the nodule, two to three cell layers outside the zone of infected cells (Figure 3B). Some intercellular spaces inside the central infected zone also became labelled. Similarly intercellular spaces of uninfected roots were clearly labelled (Figure 3C), but root cap slime and root cap cells were not labelled by any of these antibodies (data not shown).

Electron microscopy confirmed the distribution of antigens observed by light microscopy (Figure 4A−C). The matrix material surrounding bacteria in the infection threads and infection droplets was specifically and heavily labelled with gold particles, confirming an earlier preliminary study using MAC 204 (Bradley *et al.*, 1988). Moreover, the plasma membrane was also labelled, particularly in regions of accumulation of infection thread matrix material. By contrast, no matrix material and no antigen was discernible in peribacteroid spaces, even in immature cells where the bacteria would have been only recently taken up into the plant cytoplasm from the infection droplet. Similarly, the peribacteroid membrane was never observed to be labelled with any of the antibodies. In addition, label was also localized over the matrix present in some intercellular spaces, as distinct from the cell wall itself. Labelling of Golgi bodies was only observed with MAC 265, which sparsely labelled peripheral vesicles associated with the Golgi stack, probably at the secretory face (Figure 5).

As a contrast to the labelling of the infection thread matrix with MAC 204, 236 and 265, the monoclonal antibody JIM 5 was used to label sections of pea nodule tissue (Figure 4D−F). JIM 5 recognizes polygalacturonic acid, a major component of pectin molecules. The primary cell walls showed a high pectin content in recently divided cells near the nodule meristem. However, in older, non-dividing cells, the label was largely restricted to the middle lamella. At three-way junctions between cells, the distribution of the JIM 5 antigen (pectin) did not overlap with that of MAC 236

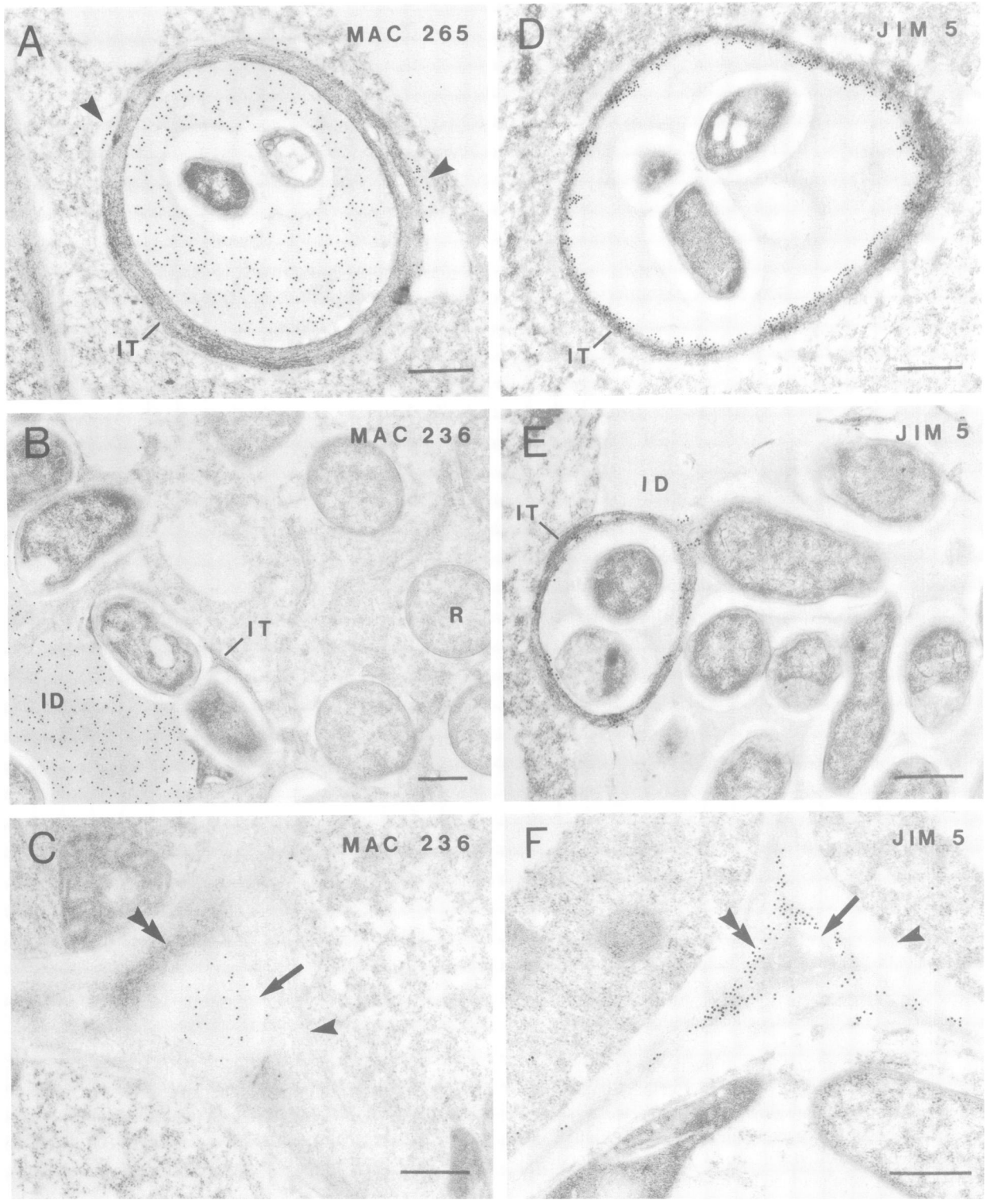

Fig. 4. Thin sections of pea nodules after immunogold labelling with monoclonal antibodies reacting with infection thread glycoprotein (MAC 236 and MAC 265) or polygalacturonic acid (JIM 5). Bars = 0.5 μm. (**A**) Labelling of the infection thread matrix with MAC 265. Note the accumulations of matrix material (arrowheads) along the plasma membrane adjacent to the infection thread (IT) 22 000 ×. (**B**) After release from an infection thread and infection droplet (ID), rhizobia (R) enclosed by peribacteroid membrane are no longer surrounded by the infection thread glycoprotein, as identified by MAC 236. 16 500 ×. (**C**) Presence of the MAC 236 antigen in an intercellular space (arrow). The antigen is not detectable in the cell wall (arrowhead) or middle lamella (double arrowhead). 23 000 ×. (**D**) Demonstration of polygalacturonic acid in an infection thread wall using JIM 5 antibody. 22 000 ×. (**E**) Following release of bacteria into the infection droplet, the JIM 5 antigen cannot be detected around the bacteria. 24 000 ×. (**F**) The middle lamella (double arrowhead) is heavily labelled with JIM 5, but the intercellular space (arrow) and cell wall (arrowhead) are free of label. 27 000 ×.

antigen (i.e. the 95K matrix component): whereas pectin, as detected by JIM 5, marked the outer zone of the cell wall, the 95K components identified by MAC 236, MAC 204 and MAC 265 were present as a matrix, filling the triangular intercellular space. Similarly, JIM 5 caused heavy labelling of infection thread walls, but matrix material of infection

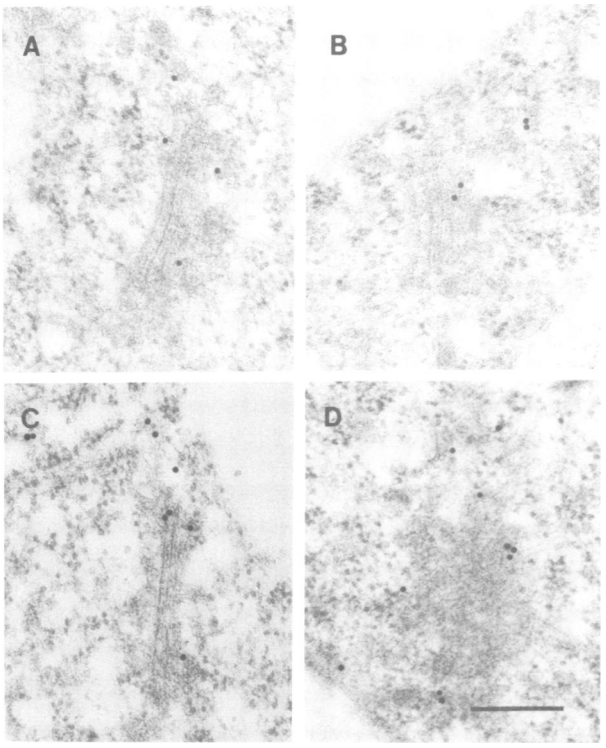

Fig. 5. Four views of Golgi bodies in pea root nodules following immunogold labelling with MAC 265. 50 000 ×. Bar = 0.25 μm. The label tends to occur at the periphery of the Golgi stack, or on associated vesicles. (**A, B, C**) Cross-sectional views of Golgi bodies. (**D**) Face view.

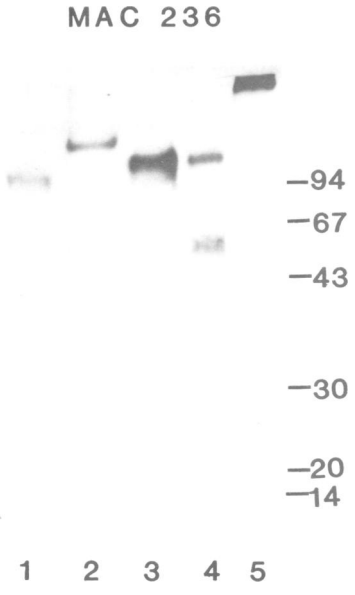

Fig. 6. Detection of the MAC 236 antigen in legumes and in carrot, as revealed by one-dimensional electrophoresis and immunoblotting. **Lanes 1, 2** and **3** represent the antigen in pea, bean and soybean nodules, respectively. **Lane 4**, secreted proteins from carrot suspension culture supernatant. **Lane 5**, Gum arabic from *Acacia*.

threads and infection droplets was not labelled with JIM 5, indicating a lack of this pectic component in the matrix. At a later developmental stage when bacteria were released from infection threads into infection droplets, the intensity of cell wall labelling by JIM 5 was dramatically reduced, although the matrix material was still strongly labelled by MAC 236.

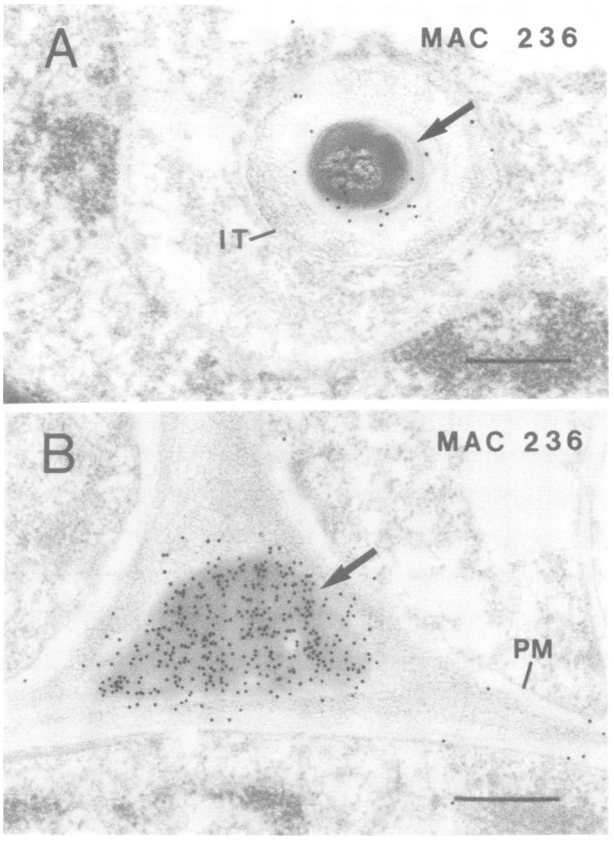

Fig. 7. Immunogold labelling of the MAC 236 antigen on thin sections of soybean nodules. 28 000 ×. Bars = 0.5 μm. (**A**) The infection thread matrix (arrow), although less abundant in soybean than in peas, is shown to contain the MAC 236 antigen. (**B**) Abundant labelling by MAC 236 of the matrix (arrow) of an intercellular space. Note the slight labelling associated with the plasma membrane (PM) in the cells bordering the space.

Occurrence of the MAC 236 epitope in other plant species

Protein preparations from *Phaseolus* bean and soybean nodules were fractionated by SDS−PAGE, transferred to nitrocellulose sheets and examined by immunostaining with MAC 236. The bands detected were similar to those obtained from pea nodules, but of slightly different mobility (Figure 6). When MAC 236 was used as an immunocytochemical probe, the matrix of infection threads and intercellular spaces was labelled in soybean nodules (Figure 7), as observed for pea. The contents of the peribacteroid space were not labelled by MAC 236 in any of the preparations from soybean, *Phaseolus* or pea.

Glycoproteins isolated from the medium of carrot suspension cultures also carried the MAC 236 epitope. The antibody recognized two bands, one near 100K, and a diffuse band at roughly 50K (Figure 6). This observation indicates that the MAC 236 epitope is not restricted to legumes, but occurs on extracellular glycoproteins in unrelated species. However, it is not known whether either protein band recognized in the carrot preparation was related to the band detected in legume nodules. Lastly, because Gum arabic (from *Acacia senegal*) had been shown to react with all three antibodies MAC 204, 236 and 265, this antigen was also examined by SDS−PAGE and immunoblotting, to see whether a low mol. wt antigen could be identified, but the antigen was shown to be of very high mol. wt and did not enter the separating gel.

Discussion

The three monoclonal antibodies MAC 204, MAC 236 and MAC 265 appear to recognize different epitopes on the 95K infection thread macromolecules (Figure 1). The two epitopes recognized by MAC 236 and MAC 265 were mutually exclusive, as seen by isoelectric focusing (Figure 2), whereas MAC 204 recognized a periodate-sensitive epitope common to both the acidic and neutral forms of 95K macromolecule. (In older root and leaf tissue from peas, MAC 204 also recognized a 40K glycoprotein: data not shown.) However, in order to establish whether the differences between neutral and acidic forms of the 95K antigen reside in the polypeptide or carbohydrate components, it will first be necessary to purify these macromolecules in substantial quantities from nodule tissue.

The extracellular distribution of the 95K antigens as visualized by immunogold labelling with MAC 236, MAC 204 or MAC 265 antibodies (Figures 4 and 7) was unique in comparison to other extracellular components localized with gold probes. The matrix glycoprotein was found in intercellular spaces outside the wall and middle lamella. It did not overlap in distribution with the pectic component of the wall identified by JIM 5, and it is clearly not an integral wall component (Fry, 1988). By contrast, JIM 5 recognized an epitope with a very similar distribution to that reported for rhamnogalacturonan I (Moore and Staehelin, 1988): both antigens were localized in the middle lamella and were especially pronounced at three-way junctions between cells. On the other hand, the extracellular glycoprotein extensin was detected in the cell wall but not in the middle lamella or intercellular spaces (Stafstrom and Staehelin, 1988), and a similar distribution was also shown for the hemicellulose xyloglucan (Moore and Staehelin, 1988).

The infection thread matrix component identified by MAC 204, MAC 236 and MAC 265 is a nodule-enhanced protein, but it is not a nodulin in the sense defined by Legocki and Verma (1980), because it also occurs in uninfected roots (Figure 1), where it is restricted to intercellular spaces (Figure 3). Such intercellular spaces are created where a cell plate fuses with the parent cell wall (Jeffree *et al.*, 1986). This space between three or more cells is lined with pectic components (Moore and Staehelin, 1988; this study), and it is likely that its formation involves the localized action of cell wall degrading or loosening enzymes (Jeffree *et al.*, 1986).

There are obvious similarities between the structure and formation of these intercellular spaces occurring at three-way junctions between cells and the structure and formation of the infection thread which is also frequently initiated at a three-way cellular junction between, for example, a curled root hair cell and two adjacent epidermal cells (Turgeon and Bauer, 1985). Both structures are bounded by pectic cell wall components and contain the same soluble matrix material. A very localized role for cell wall disruption and/or degrading enzymes has been postulated to explain how, during plant cell division, the maturing cell plate fuses with the mother cell wall (Jeffree *et al.*, 1986). Similarly, cell wall disruption and/or degradation has been postulated to explain the inception of infection thread formation in the curled root hair cell (Callaham and Torrey, 1981), and also the mechanism by which the infection thread exits through the opposite wall of the root hair cell, releasing rhizobia into the intercellular space in the sub-epidermal root cortex (Turgeon and Bauer, 1985). As the infection thread spreads from cell to cell creating an intracellular tunnel through the developing nodule cortex, a similar process of cell wall disruption and/or degradation must accompany each cycle of entry into, and exit from the cortical cells. It is thus interesting to speculate that a common component of the infection thread matrix and the intercellular space might prove to be a cell wall degrading or loosening enzyme (Fry, 1988; Cassab and Varner, 1988).

In the Ulmaceous plant *Parasponia* (the only non-legume known to form a symbiosis with *Rhizobium*), the rhizobia enter the root through a break in the epidermis at the site of *Rhizobium*-induced cortical cell proliferation (Lancelle and Torrey, 1984). Similarly, in the more primitive legume associations, the primary invasion of rhizobia into the plant roots is by an intercellular infection (Torrey, 1988). For example, in peanut (*Arachis*), infection occurs at the point of emergence of a lateral root (Chandler, 1978). In both *Parasponia* and *Arachis* the bacteria enter and proliferate in the intercellular spaces of the root cortex, surrounded by an extracellular matrix. Subsequently, infection threads develop within these cortical cells, and this in turn leads to the spread of rhizobia from cell to cell, and also in most legumes to the release of bacteria as endosymbiotic nitrogen-fixing bacteroids (Torrey, 1988). In legumes such as pea and soybean, the root hair infection thread may represent a more specialized proliferation of intercellular space which conveys the invading rhizobia through the root epidermis by creating an apoplastic (extracellular) tunnel into the root hair cell. The pre-existing mechanism for creating passages between cells may thus have been exploited by the symbiosis for the establishment of an infection thread.

It will be interesting to examine whether and how the synthesis of the macromolecules recognized by MAC 204, 236 and 265 is stimulated by close contact between plant cells and infecting rhizobia, and how material originating from Golgi bodies (Figure 5) is specifically targetted into the infection thread matrix (or the intercellular space). As regards the function of these macromolecules, it will be important to investigate how they interact biochemically with other components of plant cell walls (Fry, 1988), the bacterial cell surface and other, as yet unidentified, components of the infection thread matrix.

Materials and methods

Biological materials

Peas (*Pisum sativum* L. cv. Wisconsin Perfection) were grown as previously described (Brewin *et al.*, 1983), and inoculated with *Rhizobium leguminosarum* bv. *viceae* strain 3841 (Brewin *et al.*, 1985). Soybeans (*Glycine max* (L.) Merr. cv. Pride) were inoculated with *Bradyrhizobium japonicum*, strain USDA 110, and beans (*Phaseolus vulgaris* L. cv. Tendergreen) were inoculated with *Rhizobium leguminosarum* bv. *phaseoli*, strain 8002. Both soybeans and beans were grown as previously described for beans (Borthakur *et al.*, 1986). Nodules were isolated from plants 3–4 weeks after germination and used for immunochemical analysis.

Young nodules were harvested 3–4 weeks after germination and fractionated using differential centrifugation on sucrose cushions (Brewin *et al.*, 1985). The nodule cytoplasmic supernatant fraction was obtained after homogenization of nodules in buffer (50 mM Tris–HCl, pH 7.5; 10 mM dithiothreitol; 0.5 M sucrose; 5 mM *p*-aminobenzamidine; 5% (w/v) insoluble polyvinylpyrrolidone) and centrifugation in an Eppendorf microfuge at 10 000 *g* for 1 min. Cytoplasmic proteins from uninoculated roots were similarly obtained by homogenization and centrifugation from the apical portions of primary and lateral roots harvested 10 days after germination.

The supernatant fractions of the nodule and root preparations were used for screening the antibodies by Western blotting.

The extracellular glycoprotein derived from the conditioned medium of a carrot (*Daucus carota*) cell suspension culture was prepared by precipitation with 50% (v/v) ethanol. Gum arabic and other chemical reagents were obtained from Sigma (Poole, UK) unless otherwise stated.

Monoclonal antibodies

The rat monoclonal antibodies used in this study are listed in Table I. The derivation of MAC 204 has been previously described (Bradley *et al.*, 1988). It involved myeloma cell line Y3 Ag1.2.3 (Galfre *et al.*, 1979) and spleen cells from a LOU/iap rat that had been immunized with material released by osmotic shock treatment of peribacteroid-membrane-enclosed bacteroids. (It is probable that infection droplet structures co-purified with membrane-enclosed bacteroids during the nodule fractionation procedure, and this would account for the presence of MAC 204 antigen in the 'peribacteroid' fraction.) MAC 236 is a similar cell line obtained from the same fusion, and MAC 265 was obtained from a similar fusion performed subsequently with a LOU/C rat.

JIM 5 was raised following immunization of a LOU/C rat with protoplasts prepared from carrot suspension cells. The myeloma line was IR 983 F (Bazin, 1982). Using apple pectin (Sigma P2157) as antigen on microtitre plates (500 μg/ml in phosphate-buffered saline, incubated overnight at 4°C), a 200-fold dilution of JIM 5 hybridoma cell culture supernatant gave 90% maximal binding. Binding was 50% inhibited by 0.8 μg/ml polygalacturonic acid, or 6 mM D-galacturonic acid, or 100 mM D-glucuronic acid. L-rhamnose, L-arabinose, D-glucose, D-xylose, L-fucose, or D-galactose gave no inhibition at 100 mM.

MAC 83 is a rat IgM from the Monoclonal Antibody Centre (AFRC Institute of Animal Physiology and Genetics, Babraham, Cambridge, UK). It recognizes the CD2 antigen on porcine T-lymphocytes and, as it does not cross-react with root nodule tissue, it was used as a negative control in Western blot and immunocytochemical assays.

Affinity purification of MAC 236 antigen

The MAC 236 antibody (30 mg protein) was coupled to 5 ml of cyanogen bromide-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions, following purification of antibody by precipitation from ascitic fluid made 40% saturating with ammonium sulphate. This precipitate was resuspended in phosphate-buffered saline. The affinity column was loaded with a high speed (80 000 *g*) supernatant fraction derived from nodule homogenates (10 ml), which had been partially purified by fractionation with ammonium sulphate (30–40% saturating), and was premixed (1:1) with phosphate-buffered saline containing 1% (v/v) NP40. The column was washed under gravity with PBS 0.5% v/v NP40. After 10 column-volumes, eluting buffer was applied as recommended by the manufacturer (0.2 M glycine, 0.5 M NaCl, pH 2.8 + 10% (v/v) dioxane) and fractions (1 ml) were collected.

SDS–PAGE gels and Western blots

Proteins were separated by SDS–PAGE (Laemmli, 1970) using 12% acrylamide mini-gels, with 5 μg of protein loaded per lane. The proteins were then transferred electrophoretically (Towbin *et al.*, 1979) to nitrocellulose sheets (Schleicher and Schuell, Dassell, FRG) for 16 h at 10 V in 25 mM sodium phosphate buffer pH 6.8 (Bittner *et al.*, 1980) using a Biorad 'Transblot' apparatus (Watford, UK), or with a semi-dry blotter (Sartorius, Göttingen, FRG) using the methods and buffers of Kyhse-Anderson (1984). After blotting, the nitrocellulose sheets were incubated sequentially with blocking solution, primary antibody, a goat anti-rat alkaline phosphatase conjugate and chromogenic substrates, as previously detailed (Bradley *et al.*, 1988).

Two-dimensional gels were run as described by O'Farrell (1975). Acrylamide gel mix for the first dimension contained urea (9.5 M), acrylamide 4% (w/v), NP40 (2% v/v), ampholines (Pharmacia, Milton Keynes, UK) pH 3.5–10 (3% v/v) and ampholines pH 5–7 (2% v/v): this was polymerized in 10.5-cm tubes. After electrophoresis, the gels were electroblotted to nitrocellulose in the normal way.

Analysis of epitopes

To ascertain the carbohydrate nature of the epitopes recognized by MAC 204, MAC 236 and MAC 265, proteins transferred to nitrocellulose were oxidized by periodate treatment prior to immunostaining, according to the procedure of Woodward *et al.* (1985). Nitrocellulose sheets were incubated for 1 h in 20 or 200 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5), or in buffer alone, followed by 30 min in 50 mM sodium borohydride in Tris-buffered saline (pH 7.5). ELISA assays were performed in microtitre plates as previously described (Bradley *et al.*, 1988). Protease treatment of antigens prior to gel electrophoresis was modified from a previous procedure (Brewin *et al.*, 1985). Protein preparations (2 mg/ml) were incubated overnight at 28°C with 0.5% Triton X-100 (v/v) and proteinase K (0.1 mg/ml). As a control, proteinase K was omitted. Protease digestion was arrested by boiling samples for 3 min in Laemmli solubilization buffer (Laemmli, 1970).

Microscopy and immunocytochemistry

Pea and soybean nodules and pea root tips were fixed in glutaraldehyde (2.5% w/v in 0.2 M sodium cacodylate buffer, pH 7.0), dehydrated in ethanol and embedded in LR White acrylic resin (Agar Aids, Stansted, UK) at low temperature, and immunolabelled as described previously (Bradley *et al.*, 1988). For electron microscopy, thin sections mounted on gold grids were incubated in diluted hybridoma culture supernatant, followed by goat anti-rat gold (15 nm particles obtained from Janssen Life Sciences Products, Wantage, UK), and finally post-stained with uranyl acetate and lead citrate. The procedure for labelling of semi-thin sections mounted on glass slides was modified from that of VandenBosch (1986), using IntenSE II silver developer (Janssen Life Sciences Products) to enhance the gold labelling for light microscopy by creating an opaque black precipitate. Labelled semi-thin sections were counterstained with azure II and methylene blue in sodium metaborate, and were photographed with Ilford Pan F film. Controls for non-specific labelling included omission of the primary antibody and substitution of the irrelevant antibody MAC 83 for the primary antibody.

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