The EMBO Journal vol.4 no.3 pp.605–611, 1985

Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea cross-react with plasma membranes and Golgi bodies

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Communicated by D.A.Hopwood

Three rat hybridoma lines that produced monoclonal antibodies reacting with the peribacteroid membrane from *Pisum sativum* were isolated, and these all appeared to recognise the same antigenic structure. Using one of these monoclonal antibodies, AFRC MAC 64, electron microscopy of immunogold-stained thin sections of nodule tissue revealed that the antigen, present in the peribacteroid membrane, was also found in the plant plasma membranes and in the Golgi bodies, but not in the endoplasmic reticulum. When peribacteroid membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by electro-blotting, it was found that MAC 64 bound to a series of protease-sensitive bands that migrated in the mol. wt. range 50–85 K. The epitope was sensitive to periodate oxidation and its structure may therefore involve the carbohydrate component of a membrane glycoprotein. We suggest that this structure originates in the Golgi apparatus and is subsequently transferred to the peribacteriod membranes and plasma membranes. The monoclonal antibody also reacted with peribacteroid membranes from nodules of *Vicia* and lupin, and with plasma membranes and Golgi membranes from uninfected plant cells, including root tip cells from onion (*Allium cepa*), indicating that the antigen is highly conserved in the plasma membranes of plant cells.

Key words: Rhizobium/peribacteroid membrane/plasma membrane/monoclonal antibody/pea

Introduction

The *Rhizobium*-legume symbiosis involves a high degree of morphological and biological specialisation by both partners. Many thousand nitrogen-fixing bacteria (bacteroids) are maintained in the cytoplasm of each infected root nodule cell, and in pea nodules each bacteroid is individually enclosed by a periobacteroid membrane of plant origin (Robertson *et al.*, 1984a). The peribacteroid membrane originates during nodule morphogenesis by endocytosis from the bounding membrane of an infection thread, which was in turn formed by involution of the plasma membrane at the initial stage of infection at the surface of a root hair cell. At a later stage, within each infected cell, biogenesis of the peribacteroid membrane involves the coalescence of vesicles derived from Golgi bodies (Verma *et al.*, 1978; Robertson *et al.*, 1978a). The peribacteroid membrane represents an important interface for the exchange of metabolites between the cytoplasms of eukaryote and prokaryote; in addition, it may be involved in surface interactions with the endosymbiont which allow normal nodule morphogenesis to proceed (Robertson and Lyttleton, 1984).

To study both the function and biogenesis of molecular components of the peribacteroid membrane of pea root nodules, we have obtained monoclonal antibodies that react with antigens on the peribacteroid membrane. This experimental approach has made use of immunogold labelling to localise antigens on thin sections of legume nodule tissue prepared for electron microscopy (Robertson *et al.*, 1984b, 1985). We report here the isolation of several monoclonal antibodies that are directed against the same glycoprotein component of the peribacteroid membrane, and some cytological and biochemical properties of this antigen.

Results

Isolation of peribacteroid membrane

Figure 1A shows an electron micrograph of a membrane-enclosed bacteroid. The peribacteroid membrane appeared to be intact, and this was the case for >80% of the bacteroids in the preparation (P4). Large amounts of vesicular membrane material (the peribacteroid fraction, S6) were released from the membrane-enclosed bacteroids by mild osmotic shock treatment. This membrane material remained in the supernatant fraction after 2 min at 10 000 *g*, but after fixation with glutaraldehyde the membrane components of the fraction could be prepared by centrifugation at 10 000 *g* for 5 min (Figure 1B). The peribacteroid fraction (S6) was also compared by SDS-polyacrylamide gel electrophoresis (Figure 2) with samples of sonicated bacteroid supernatant (S10) and with the first nodule cytoplasmic supernatant (S1) which contained leghaemoglobin. The pattern of bands obtained with the peribacteroid fraction after staining the gel with Coomassie blue or with periodate and silver nitrate was relatively simple, and clearly distinct from that seen with either the bacteroid or the nodule cytosol fractions.

Isolation of monoclonal antibodies to the peribacteroid membrane

Three separate fusion experiments were performed with spleen cells isolated from rats that had been immunised with peribacteroid material. Supernatant fractions from 220 hybridoma cell lines were screened for antibody that reacted with antigens derived from the peribacteroid fraction (S6), using the dot immunoassay methods of Smith *et al.* (1984). Of 10 positive cell lines, three were identified (AFRC MAC 63, AFRC MAC 64 and AFRC MAC 65) which produced antibodies that reacted with the peribacteroid fraction but not with sonicated free-living bacteria (S11). Furthermore, all three monoclonal antibodies, when tested for immunogold localisation on thin sections of nodule tissue stained the peribacteroid membranes (Figure 3).

Immunostaining of electroblots with MAC 64

Samples of peribacteroid material (S6), bacteroids (S10) or nodule cytoplasmic supernatant (S1) were examined after SDS-polyacryl-

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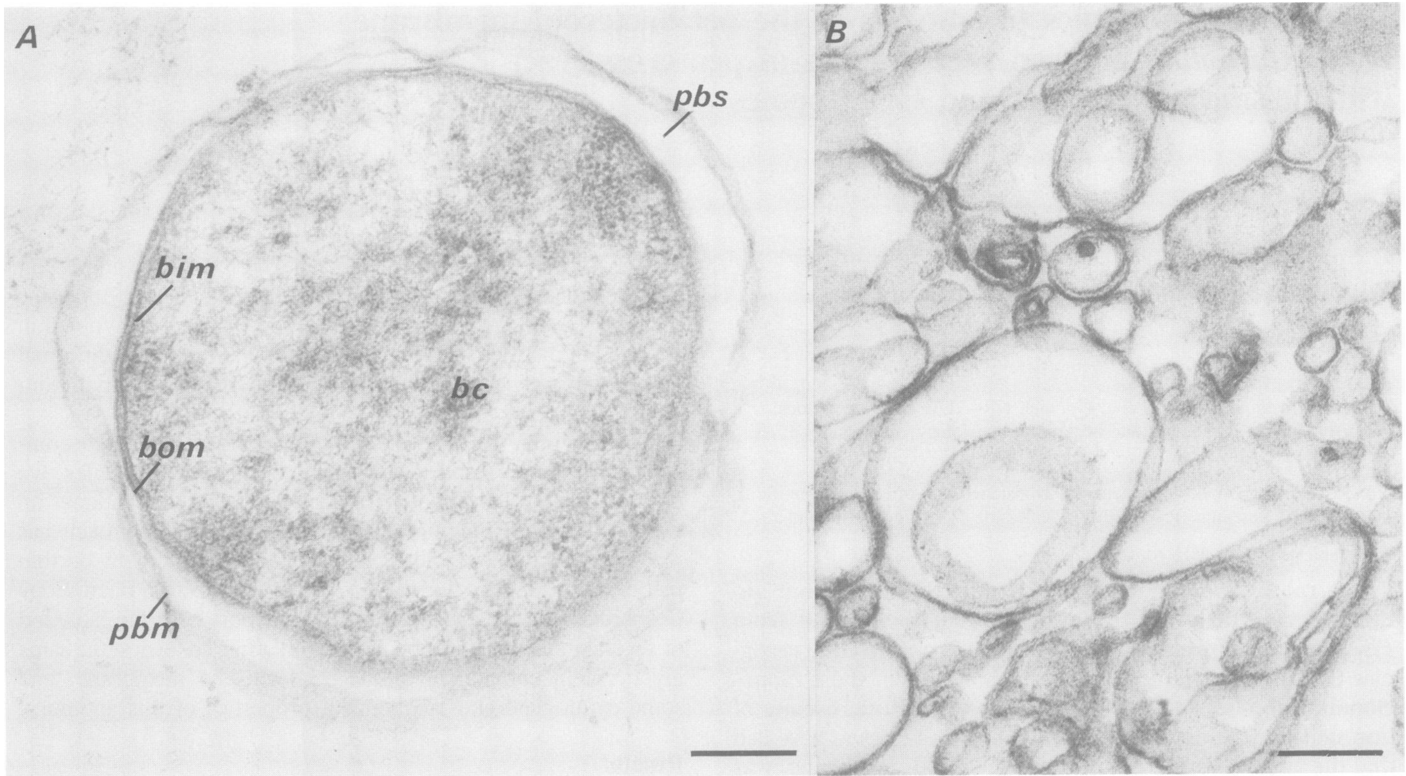

Fig. 1. **(A)** Thin section stained with uranium and lead showing a single membrane-enclosed bacteroid from preparation P4 fixed with glutaraldehyde and osmium, and embedded in LR white resin. Bacteroid cytoplasm, bc, bacteroid envelope inner membrane, bim; bacteroid envelope outer membrane, bom; peribacteroid space, pbs; peribacteroid membrane, pbm. Bar mark in this and subsequent electron micrographs represents 0.2 μm. **(B)** Thin sections stained with uranium and lead showing the content of the membrane pellet obtained after fixation and centrifugation of the peribacteroid fraction (S6).

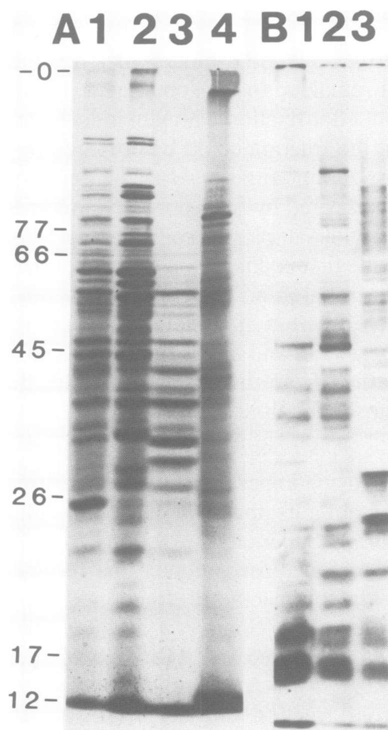

Fig. 2. Examination of fractions used as antigens by SDS-polyacrylamide gel electrophoresis. **A**, after staining with Coomassie blue. **B**, after periodate-silver staining for carbohydrates and glycoproteins. **1**, free-living *R. leguminosarum* 3841 (sonicated supernatant S11); **2**, bacteroids (sonicated supernatant S10); **3**, peribacteroid fraction (S6); **D**, nodule cytoplasmic supernatant (dialysed S1). The top of the gel (-O-) and the positions of protein mol. wt. markers (x 1000) are indicated.

amide gel electrophoresis, blotting onto nitrocellulose sheets and incubation with MAC 64 (Figure 4). In all experiments the behaviour of MAC 63, 64 and 65 was indistinguishable. The staining pattern consisted of a dispersed series of bands in the size range 50−85 K, as judged by protein mol. wt. markers. The distribution of these bands was not affected by the omission of insoluble polyvinyl pyrollidone or protease inhibitors from the extraction buffer.

MAC 64 antigen (i.e., the molecule(s) recognised by MAC 64) was also associated with the nodule cytosol supernatant fraction, S1 (Figure 4), but a supernatant fraction from sonicated free-living bacteria (S11) did not react with the antibody (data not shown). However, the supernatant from sonicated bacteroids (S10) still contained considerable amounts of MAC 64 antigen, probably because peribacteroid membrane material had co-sedimented with the bacteroids. If the bacteroids were further purified by sucrose gradient centrifugation (15−70% w/v) in a 1.5 ml microcentrifuge tube at 800 *g* for 10 min in a Denley bench centrifuge, much of this antigenic material was left at the top of the gradient, although some still remained associated with the bacteroid fraction. The size distribution of antigens reacting with MAC 64 was slightly different for peribacteroid, bacteroid and plant cytoplasmic fractions (Figure 4).

Biochemical properties of MAC 64 antigen

MAC 64 antigen appeared to be a membrane-associated glycoprotein or group of glycoproteins without detectable lipid component. This conclusion is drawn from the following observations. Treatment of the peribacteroid material (S6) with Triton X-100 (0.5% v/v) resulted in solubilisation of the antigen which otherwise could be sedimented with the membrane fraction following centrifugation for 15 min at 10 000 *g*.

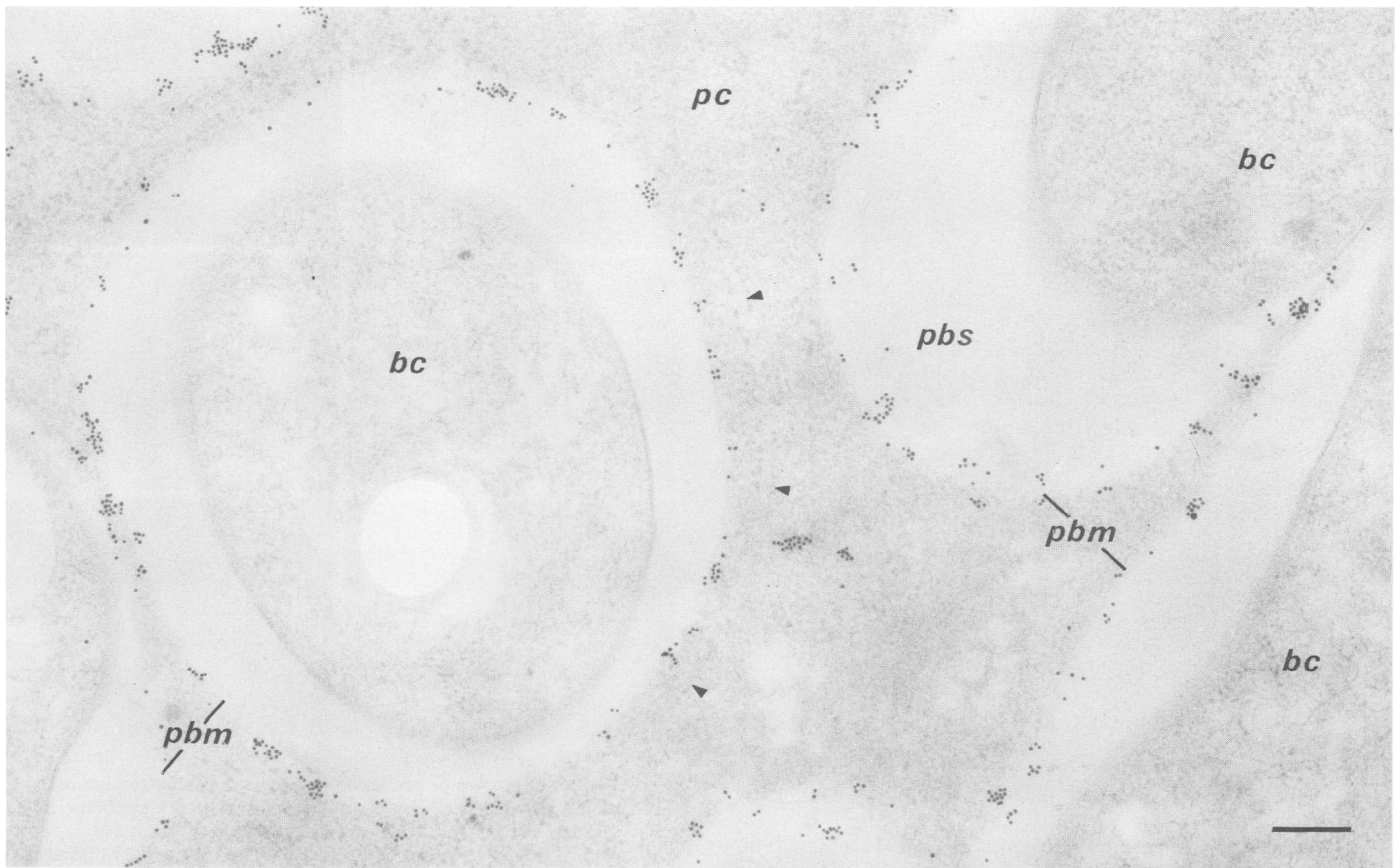

Fig. 3. Thin section of nodule tissue from a 15-day old pea nodule immunostained using AFRC MAC 64 and post-stained with uranium and lead. Specific immunogold staining was only observed on the peribacteroid membrane (pbm). No staining was observed on the endoplasmic reticulum (arrow heads), bacteroid cytoplasm (bc) or plant cell cytoplasm (pc).

Immunogold labelling of thin sections of nodule tissue was abolished by the treatment with sodium periodate-HCl (Figure 5), indicating the presence of glycosidic groups in MAC 64 antigen. In a control experiment, leghaemoglobin could still be detected using anti-leghaemoglobin antiserum. Similarly, after gel electrophoresis and electroblotting to nitrocellulose sheets the binding of MAC 64 to antigen was eliminated by a pre-treatment with periodate.

The presence of a protein component in the antigen was indicated by the fact that pronase treatment, prior to gel electrophoresis and electroblotting, entirely eliminated the antigen (Figure 4). Similarly, treatment with trypsin resulted in loss of all bands >76 K. However, when the pronase treatment was applied to nodule thin sections fixed and embedded in Lowicryl K4M resin, gold labelling of antigen in the peribacteroid membrane was still observed with MAC 64 (Figure 5), although no reaction for leghaemoglobin was obtained using anti-leghaemoglobin antibody. Assuming that the pronase treatment would have digested any exposed polypeptide chains, this result indicates that the polypeptide component may not contribute to the antigenic site which is recognised by MAC 64.

In an attempt to identify the MAC 64 antigen in a lipid component, peribacteroid material was extracted with organic solvents. Although the antigen was identified by dot immunoassay in the aqueous layer and at the interface, it was not found in the organic phase, suggesting that the antigen was not a glycolipid. However, we have not yet excluded the possibility that the antigen could contain a small covalently attached lipid side chain.

Tissue distribution of MAC 64 antigen

Using the immunogold localisation technique, MAC 64 antigen was identified in the peribacteroid membrane of pea nodule cells (Figure 3). The antigen could also be identified on the plasma membrane of infected cells (Figure 6), and on the infection thread membrane. The antigen was also detected in the membrane of the Golgi apparatus in pea and *Vicia* (Figure 6), but not in any of the other cell membrane systems (endoplasmic reticulum, mitochondrial or nuclear membranes), nor in the plant cell cytoplasm. The antigen was not restricted to infected cells, being also present on the surface membrane of cells from the pea root tip and of root hair cells. Nor was it confined to peas: it was also detected on peribacteroid and plasma membranes from lupin nodule cells, and in the Golgi membranes from onion root meristem cells (data not shown).

Discussion

The use of monoclonal antibodies for tissue localisation of antigens in plants is not as advanced as in animal systems and the present report is one of the first for a monoclonal antibody that is specific for a particular class of plant membrane. The fact that MAC 64 reacted with a component of the peribacteroid membrane was only demonstrated conclusively (Figure 3) by using the technique of immunogold localisation on thin sections of nodules (Robertson *et al.*, 1984b). Because this protocol is relatively simple, and large numbers of thin sections can be derived from a single block of fixed and embedded tissue,

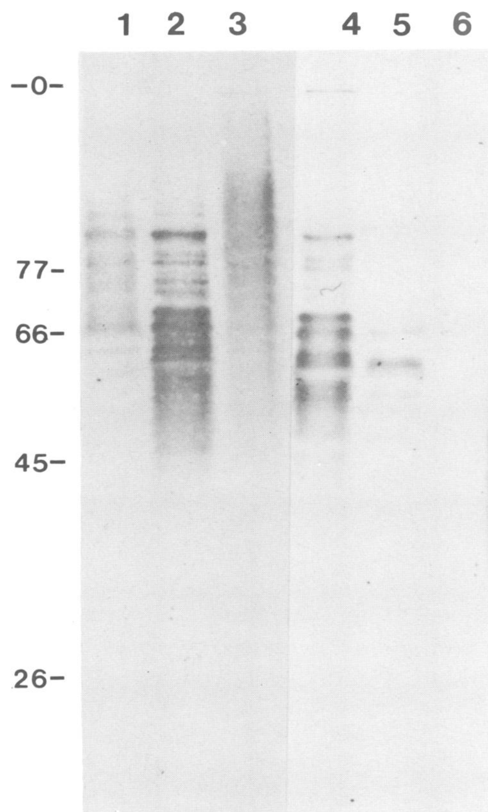

Fig. 4. Identification of antigens reacting with monoclonal antibody AFRC MAC 64 following SDS-polyacrylamide gel electrophoresis, electroblotting to nitrocellulose sheets and staining by the indirect immunoperoxidase technique. **(A) 1**, Bacteroids (sonicated supernatant S10); **2**, peribacteroid fraction (S6); **3**, nodule cytoplasmic supernatant (dialysed S1). **(B)** Effects of pre-digestion of peribacteroid fraction (S6) prior to gel electrophoresis: **4**, untreated; **5**, trypsin (1 mg/ml); **6**, pronase (1mg/ml).

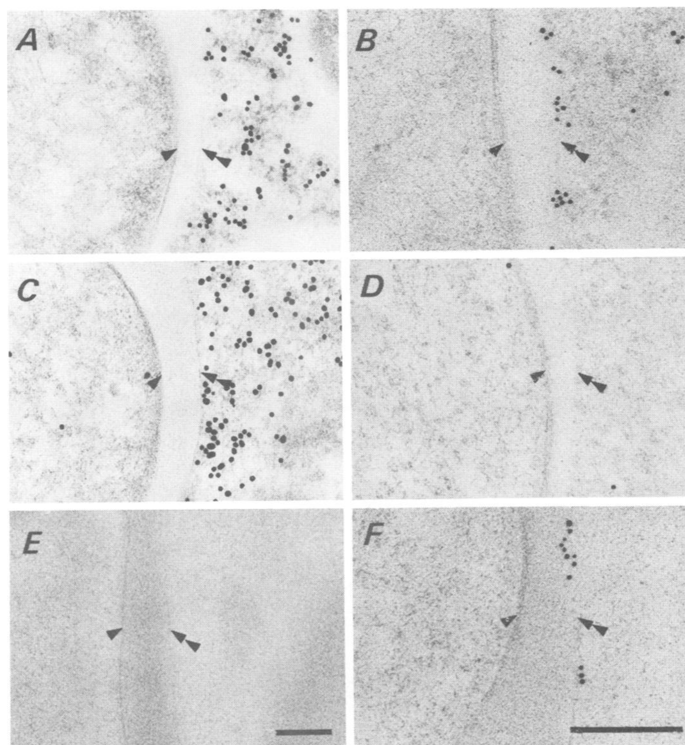

Fig. 5. Effect of pronase or periodate pre-treatments of thin sections of nodule tissue showing small portions of bacteroids and plant cytoplasm. In each case the bacteroid envelope outer membrane (arrow head) and peribacteroid membrane (double arrow head) is labelled. **A, C, E.** Sections immunogold stained using antibody to leghaemoglobin. **B,D,F.** Sections immunogold stained using antibody (AFRC MAC 64) to the peribacteroid membrane. Sections **C** and **D** were pre-treated with periodate (which eliminated binding of MAC 64 in **D**). Sections **E** and **F** were pre-treated with pronase (which inhibited binding of anti-leghaemoglobin antibody in **E**).

immunogold localisation can be used as a general screening system to pinpoint the site of reactivity of any monoclonal antibody within a tissue section.

Evidence from periodate oxidation and protease treatments (Figures 4 and 5) indicated that the epitope for MAC 64 almost certainly involves the carbohydrate moiety of a glycoprotein, or group of glycoproteins, of dispersed mol. wt. in the range 50−85 K on SDS-polyacrylamide gels. Failure of pronase treatment to eliminate the binding of MAC 64 to thin sections (Figure 5) could be due to the protection of protein moieties within membrane or by the embedding resin material. Alternatively, glutaraldehyde fixation during the embedding procedure could have made the glycoprotein resistant to proteolysis.

The dispersed size range for the antigen molecules seen on SDS gels (Figure 4) could result from one or a combination of the following: different levels of carbohydrate substitution on a single polypeptide; substitution of the same carbohydrate moiety on several different polypeptides in the same membrane system (Goldberg and Kornfeld, 1983); or partial proteolysis. These same mechanisms could explain the slight variations in size-distribution for MAC 64 antigens from peribacteroid, bacteroid and plant cytoplasmic fractions (Figure 4).

The major antigenic compound present in the peribacteroid fraction was the membrane glycoprotein to which MAC 64 reacted. In addition to MAC 63, 64 and 65, which were derived from two rats, we isolated three more monoclonal antibodies from another fusion experiment with a third animal (data not shown), and these also reacted to the same epitope (as judged by immunoblotting and immunocytochemistry). Thus, six out of 20 positive cell lines were of the same specificity. Therefore, this antigenic determinant was either very abundant or highly immunogenic, or both. [Similarly, Anderson *et al*, (1984) found that the carbohydrate components of plant glycoproteins were highly immunogenic in mice.] Alternatively, our screening assay was biased towards the detection of this antigen, for reasons unknown. To obtain monoclonals to other components of the peribacteroid fraction, which are clearly visible on SDS gels (Figure 2), it may first be helpful to use a MAC 64 affinity column to remove this antigen from the peribacteroid fraction before immunising the rats. A similar procedure was employed to remove bacterial lipopolysaccharide from the peribacteroid fraction used as antigen in the present study.

Immunogold labelling of thin sections of nodule tissue showed that these monoclonals, which reacted with the peribacteroid membrane, also reacted with antigens on the plant cell plasma membrane and in the Golgi bodies (Figure 6). As discussed above, the constituent recognised by MAC 64 is most probably the carbohydrate side chain of a glycoprotein, which could have originated in the Golgi bodies (Figure 7) where glycosylation reactions of plant proteins are known to occur (see Herman and Shannon, 1984). Previous cytochemical evidence (Robertson *et al.*, 1978a), showed that the plasma membrane and peribacteroid membranes are related (staining with phosphotungstic acid or silver, but not with zinc iodide-osmium tetroxide); however these membranes were indistinguishable from those of the Golgi body

Fig. 6. (A) Thin section of non-infected plant cell from a root of *Vicia hirsuta*, showing immunogold staining of the plasma membrane (pm) with AFRC MAC 64. Cell wall, cw, plant cytoplasm, pc, and the vacuole, v, show no specific staining. **B,C,D**. Thin sections of *Vicia* nodules from 6-day old plants showing immunogold staining of the Golgi bodies. The staining tends to occur towards the outer edges of the cristae or towards one face of the Golgi bodies.

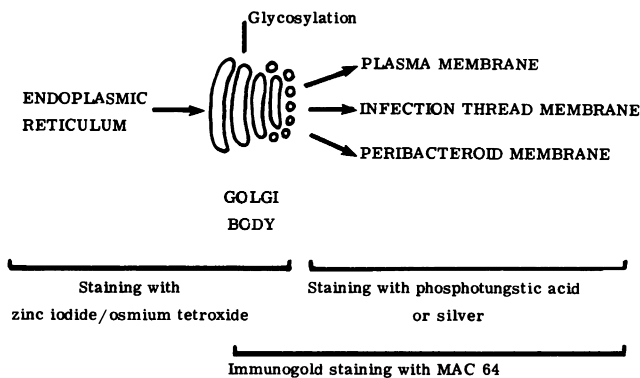

Fig. 7. Model for the biogenesis of peribacteroid membrane, showing the distribution of immunogold stained MAC 64 antigen relative to cytochemical staining (Robertson *et al.*, 1978a) with zinc oxide and osmium tetroxide; phosphotungstic acid; and silver.

and endoplasmic reticulum (which each stained with zinc iodide-osmium tetroxide, but not with phosphotungstic acid or silver). It was suggested that the distal (*trans*) face of Golgi bodies is a transition zone between the endoplasmic reticulum and the cytoplasmic vesicles that are directed either towards the peribacteroid membrane or the plasma membrane. The distribution of material reacting with MAC 64 agrees with this general picture (Figure 7). The immunogold particles tended to occur towards the outer edges of the cisternae or towards one face of the Golgi bodies (Figure 6), but it was not clear whether this staining was on the proximal (*cis*) or distal (*trans*) face.

The membrane glycoprotein identified in this study is not a nodulin as defined by the immunological studies of Legocki and Verma (1980) and Bisseling *et al.* (1983), because it is not an antigen that is found exclusively in the tissues of root nodules, being present in the Golgi vesicles of uninfected pea root meristem cells and the plasma membrane of root hair cells. It was also identified in the plasma membranes and Golgi membranes

from onion root tip cells. Thus, the monoclonal antibody MAC 64 should prove useful in characterising the maturation and exchange of material between the Golgi apparatus and the plasma membrane of plant cells in general, quite apart from any specific applications as an immunochemical marker for the peribacteroid membrane in further studies on the *Rhizobium*-legume interaction.

Materials and methods

Biological materials

Peas (*Pisum sativum* var. Wisconsin Perfection) were grown as described previously (Brewin *et al.*, 1983) in cabinets at 20°C and 150 μ Einsteins/m^2. *Rhizobium leguminosarum* strain 3841 was used for inoculation and cultured on agar slants containing rich medium (Wang *et al.*, 1982). Nodules from lupin (*Lupinus angustifolius* cv. uniwhite) and *Vicia hirsuta* were formed by infection of roots with *R. lupini* NZP 2257 and *R. leguminosarum* 8401 pRL1J1, respectively. Onion roots (*Allium cepa*) were taken from 3-day old seedlings germinated in the dark on damp tissue paper. Nodules from 100 pea seedlings were harvested ~2 days after the onset of N_2 fixation (normally 14 days after inoculation).

Biochemical fractionation of root nodules

The preparation of membrane-enclosed bacteroids, i.e., those surrounded by an intact peribacteroid membrane, was modified from published methods (Bisseling *et al.*, 1983; Robertson *et al.*, 1978b). All procedures were carried out at 4°C, and the homogenisation buffer contained 5% (w/v) insoluble polyvinyl pyrollidone (P6755), and a protease inhibitor (5 mM *p*-amino benzamidine), to prevent possible modification of proteins during extraction. (All reagents were obtained from Sigma.) Approximately 10 g of nodules were homogenised in a pestle and mortar in 10 ml Tris-DTT buffer (50 mM Tris-HCl, pH 7.5; 10 mm dithiothreitol) containing 0.5 M sucrose. The homogenate was filtered through Miracloth (Calbiochem), transferred to 8 x 1.5 ml microfuge tubes and centrifuged on an Eppendorf microcentrifuge 5414 at 10 000 *g* for 1 min. All subsequent centrifugations were carried out in Eppendorf centrifuge tubes at 10 000 *g*. The nodule cytoplasmic supernatant (S1) was removed and held on ice: the pellets (P1), which contained membrane-enclosed bacteroids, were gently resuspended in a total volume of 1.5 ml Tris-DTT buffer containing 0.5 M sucrose, using a Pasteur pipette so that starch grains remained as a compact residue at the bottom of the tube. Aliquots (0.5 ml) of this suspension were layered over a 0.6 ml cushion of 1.2 M sucrose in Tris-DTT buffer and centrifuged for 30 s to remove any remaining starch grains and cell debris. The interface and all of the 0.5 M sucrose layer was removed and centrifuged for 1.5 min. The pellet (P2) was gently resuspended in 3 ml of 0.5 M sucrose in Tris-DTT buffer and recentrifuged for 1 min. The pellet (P3) was resuspended in 1.5 ml of 0.5 M sucrose in Tris-DTT buffer, and aliquots (0.5 ml) were layered over 0.6 ml 1.0 M sucrose in Tris-DTT buffer and centrifuged for 5 min. The resulting pellet (P4) consisted mainly of bacteroids enclosed by peribacteroid membrane. This material was resuspended in 1.0 ml Tris-DTT buffer without sucrose and then centrifuged for 2 min. The supernatant fraction (S5), containing crude peribacteroid material, was centrifuged for 1 min to remove residual bacteroids and the supernatant, called the peribacteroid fraction (S6), was retained. (This fraction contained peribacteroid membrane and material from the peribacteroid space as well as some broken bacteroids.) After the osmotic shock treatment, the bacteroids (P5) were washed twice with 2 ml Tris-DTT buffer (no sucrose), centrifuged for 2 min and then washed twice with 2 ml Tris-DTT buffer containing 0.5 M sucrose to give a pellet of washed bacteroids (P9). The preparation of peribacteroid material (S6) was stored at −20°C, unless used immediately. Bacteroids (P9) were subsequently disrupted by sonication, centrifuged for 1 min at 10 000 *g* in an Eppendorf tube and this bacteroid supernatant fraction (S10), which contained bacteroid cell envelopes as well as soluble cytoplasmic proteins, was stored at −20°C or used directly.

Free-living bacteria were harvested from 3 day slants, washed twice in 50 mM Tris-HCl pH 7.4, 200 mM NaCl, disrupted by sonication, centrifuged for 1 min at 10 000 *g*, and the rhizobial supernatant fraction (S11) was retained.

Derivation of monoclonal antibodies to the peribacteroid membrane

Two female AO rats were injected i.m. with 200 μl of peribacteroid material (fraction S6) containing ~100 μg protein (Lowry *et al.*, 1951) in complete Freund's adjuvant. This immunisation was repeated after 4 and 8 weeks but using subcutaneous (s.c.) injections of antigen in incomplete Freund's adjuvant (IFA). After 16 weeks the rats were re-inoculated s.c. with peribacteroid material (100 μg protein in IFA) that had been passed through an immunoaffinity column containing CH-Sepharose 4B coupled to the monoclonal antibody AFRC MAC 57 (Robertson *et al.*, 1985) which binds to and removes bacterial lipopolysaccharide. (A detailed description of AFRC MAC 57 and the preparation of the immunoaffinity column will be published elsewhere.) After 22 weeks a further i.v. injection of the immuno-purified peribacteroid material in 0.85% (w/v) saline was carried out, and 3 days later spleen cells were fused with the rat myeloma line Y3 Ag1.2.3 (Galfre *et al.*, 1979; Galfre and Milstein, 1981). Ten to 15 days after fusion, supernatants from confluent cultures were tested by dot immunobinding assay for the presence of specific monoclonal antibodies (Smith *et al.*, 1984).

In the initial screen by dot immunoassay, the antigens used were the peribacteroid fraction (S6) and a soluble preparation derived from bacteroids (S10) after sonication and centrifugation. (~0.5 μg protein was applied in each 1 μl dot of antigen.) Any culture supernatants that were positive in these screens were subsequently tested by dot immunoassay for their inability to bind to a preparation of free-living *R. leguminosarum* 3841 that had been sonicated and centrifuged in parallel with the bacteroid preparation.

Selected positive cultures were cloned by limiting dilution in flat-bottomed microtitre plates (NUNC Cat no. 1-67008), using 2000 rad-irradiated rat or mouse peritoneal cells as feeders.

Immunoperoxidase staining of antigens on nitrocellulose

Nitrocellulose sheets (Schleicher and Schull BA85) for the dot immunobinding assay were treated as described by Smith *et al.* (1984) except that, after application of the antigen, the sheets were washed for 5 min in 20% (v/v) methanol containing 10 mM HCl in order to inactivate endogenous peroxidase activity in the samples. When whole sheets were to be developed with a single monoclonal antibody (e.g., after gel electrophoresis and electroblotting) the nitrocellulose was washed in Tris-buffered saline (TBS = 50 mM Tris HCl, 200 mM NaCl, pH 7.4), incubated for 1 h in TBS containing 1% (w/v) BSA (Sigma fraction V) and then incubated overnight in this solution containing 2% (v/v) of the hybrid myeloma supernatant. Sheets were then washed five times with TBS and incubated for 2 h in TBS containing 1% BSA, together with the second antibody which was rabbit anti-rat IgG conjugated to horseradish peroxidase (Miles-Yeda code 61-206), used at 1:2000 dilution. After five washes with TBS, colour was developed using 0.6 mg/ml 4-chloro 1-naphthol (Sigma) and 0.01% (v/v) hydrogen peroxide (Hawkes *et al.*, 1982).

Preparation of samples for electron microscopy

Fixation and embedding procedures were modifications of those used by Robertson *et al.* (1978b) for peribacteroid membrane and membrane-enclosed bacteroid fractions from lupin. Samples were fixed by incubation with glutaraldehyde (final concentration 1% v/v) for 2 h at 0°C in polypropylene microfuge tubes containing 1% (v/v) glutaraldehyde in 0.25 ml, 50 mM Tris-HCl, pH 7.4 (0.5 M sucrose was also included for the preparation of membrane-enclosed bacteroids). The enclosed bacteroid and peribacteroid samples were then centrifuged for 15 min at 10 000 *g*. The supernatants were discarded and twice replaced with 25 mM sodium cacodylate buffer, pH 7.2, without disturbing the pellets which were left overnight at 4°C in 2% osmium tetroxide in cacodylate buffer. After removal of the osmium by replacement with cacodylate buffer, the samples, at room temperature, were treated successively for 30 min each with 30% and 50% (v/v) ethanol, 60 min with 70% ethanol saturated with uranyl acetate (kept in the dark), 30 min each with 80% and 90% ethanol, 1 h in 100% ethanol and finally for 2.5 h in fresh 100% ethanol. The pellets were then treated for 26 h with 100% LR white resin at 4°C in the dark. The tips of the polypropylene tubes were cut off, the pellets were cut into three pieces and transferred to fresh LR white resin for 8.5 h at 4°C. Pieces of pellets were then transferred to gelatin capsules containing fresh resin, which was polymerised by incubation at 60°C for 16 h and sectioned for electron microscopy.

Immunogold labelling of thin sections of plant tissue embedded in Lowikryl K4M resin was carried out as described by Robertson *et al.* (1984b, 1985). Nodule tissue from pea, *Vicia* and lupin were examined, and also pea, *Vicia*, lupin and onion root tissues, taken in the region of growing root hairs. A rabbit polyclonal antiserum to leghaemoglobin was a gift from T.Bisseling (Wageningen). For the primary reaction of the rat monoclonal antibodies with the antigens on the surface of thin sections, hybrid myeloma culture supernatants were diluted 1:10 in Tris-buffered saline (TBS) containing 20 mg/ml bovine serum albumin (Fraction V, Sigma). The secondary immunogold staining reaction involved the use of a 1:30 dilution of goat anti-rat IgG-Au (10) (Janssen Pharmaceutica) in 2 mg/ml bovine serum albumin in TBS. Sections were post-stained with uranium and lead.

Polyacrylamide gel electrophoresis and electroblotting

Polypeptides were separated on isotropic polyacrylamide slab gels, using the discontinuous buffer system described by Laemmli (1970). The addition to membrane samples of Triton X-100 to a final concentration of 0.5% (v/v) was found to improve the resolution of antigen bands following subsequent addition of SDS and gel electrophoresis for 10 h at 100 mV. Gels were stained for proteins with Coomassie blue (Fairbanks *et al.*, 1971) or for carbohydrates and glycoproteins using the periodate-silver staining system of Tsai and Frasch (1982). Proteins were electroblotted (Towbin *et al.*, 1979) from the gel to a sheet of nitrocellulose for 16 h at 10 V in 25 mM sodium phosphate buffer pH 6.5 (Bittner *et al.*, 1980). After electroblotting, the nitrocellulose sheets were immunoperoxidase stained as described above.

Biochemical analysis of antigens from the peribacteroid membrane

In order to determine the biochemical nature of the antigenic site that reacts with MAC 64, the peribacteroid fraction (S6) was digested with pronase (1 mg/ml, protease type XIV), or trypsin (1 mg/ml type III) for 1 h at 28°C in 50 mM Tris buffer pH 7.5 containing 0.5% (v/v) Triton X-100. (Enzymes were obtained from Sigma.) Enzymic digestions were stopped by the addition of an equal volume of SDS-PAGE solubilisation buffer and samples were then heated to 90°C for 3 min and loaded onto polyacrylamide gel in the normal way.

In further tests on the nature of the antigenic site, thin sections of nodule tissue embedded in Lowicryl K4M and supported on the surface of electron microscope grids were immersed in either pronase (1 mg/ml) in 50 mM Tris-HCl pH 7.5 for 1 h at 25°C, or for 15 min in a saturated solution of sodium metaperiodate, followed by treatment for 5 min in 0.1 M HCl. After washing with Tris buffer, the sections were carried through the normal schedule for immunogold staining (Robertson *et al.*, 1984b) using either anti-leghaemoglobin antiserum or monoclonals to the peribacteroid membrane. Periodate oxidation under similar conditions was also conducted on the antigen adsorbed to the surface of nitrocellulose sheets following gel electrophoresis and electroblotting.

Lipid extraction from the peribacteroid fraction followed the procedures of Dzandu *et al.* (1984) and Hamaguchi and Cleve (1972).

Acknowledgements

We thank Dr K.Roberts and Professor D.A.Hopwood for helpful discussions and Julia Goddard for preparing the manuscript.

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Received on 10 December 1984