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Ultrastructural Localisation of Sialoadhesin (Siglec-1) on Macrophages in Rodent Lymphoid Tissues

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Abstract

In previous studies it has been demonstrated that sialoadhesin is a macrophage-restricted adhesion receptor for lymphocytes and myeloid cells. It is under normal circumstances expressed by subpopulations of macrophages in lymphoid and haemopoietic tissues. In this study different immunoelectronmicroscopical techniques are used to investigate the ultrastructural localisation of sialoadhesin within the lymph node and spleen of rodents. The results show that sialoadhesin is selectively expressed by a subset of macrophages in peripheral lymphoid tissues. Sialoadhesin was localised predominantly on the plasma membrane and in particular in areas of intimate contact with lymphocytes, thereby visualizing putative local interaction between these cells. Interestingly, sialoadhesin was also detected in intracellular vesicles that were apparently taken up by macrophages. These findings are consistent with the putative role of sialoadhesin in local cell-cell interactions in lymphoid tissues. Surprisingly, sialoadhesin was also found at contact points of macrophages with other macrophages, sinus-lining cells and reticulum cells, suggesting that sialoadhesin also mediates interactions with these cell types.

Introduction

Sialoadhesin is a macrophage-restricted plasma membrane receptor. The molecule mediates cell-cell adhesion of lymphoid and myeloid cells, via recognition of particular α 2,3-linked sialic acid containing glycoconjugates (1–4), expressed on the cell surface. Sialoadhesin is a member of the immunoglobulin superfamily (IgSF) containing the large number of 17 extracellular Ig domains (5). The receptor is closely related to CD22, CD33 myelin-associated glycoprotein (MAG), Schwann cell myelin protein (SMP) (6, 7) and siglecs 5–9 (8) and together with these molecules forms a family of sialic binding IgSF members, recently called siglecs (9).

The tissue distribution of sialoadhesin has been studied in rodents using light microscopical techniques. Under normal circumstances sialoadhesin is expressed on a variety of tissue macrophages including resident bone marrow macrophages and on subpopulations of macrophages in the splenic marginal zone, and on macrophages in lymph node

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subcapsular sinus and medulla (10–14). Sialoadhesin is also expressed by macrophages in the adrenal gland (9). During autoimmune diseases like experimental allergic encephalomyelitis (EAE), nephritis, thyroiditis and rheumatoid arthritis the infiltrating macrophages also express high levels of sialoadhesin (15–19). Our previous ultrastructural studies in the murine bone marrow have indicated that the molecule is present on resident bone marrow macrophages and is concentrated at contacts with developing myeloid cells (20).

In this study we have investigated the ultrastructural localisation of this transmembrane glycoprotein in rodent peripheral lymphoid tissues, in relation to its potential role in local cell-cell interactions.

Materials and Methods

Animals

Male young adult Swiss mice and Wistar rats were obtained from Harlan/CPB (Zeist, The Netherlands) and kept under conventional laboratory conditions, with free access to food and water.

Antibodies

- The monoclonal antibody SER-4 a rat immunoglobulin (IgG2a) recognizing mouse sialoadhesin (1–4) has been described before.
- The mAb ED3, mouse IgG2a is directed against rat sialoadhesin (10, 12, 13).
- The mAb MOMA-1, rat anti mouse IgG2a, recognizing a subpopulation of macrophages in the subcapsular sinus (21).
- The mAb ER-TR9 (IgM), specific for mature mouse subpopulations (22).

Tissue preparation

Animal perfusion was performed via the abdominal aorta for approximately 5 min, with 0.1M phosphate buffer (pH = 7.3) using a peristaltic pump. After dissection the popliteal nodes and spleen, they were immersed in periodate-lysine-paraformaldehyde (PLP) fixative (23) containing 2% paraformaldehyde for 2 h at 4 °C. The tissue was cut in slices of 0.5 mm perpendicular to their long axes and fixed for another 2 h in PLP solution. Subsequently, the tissue blocks were washed in 0,1M PBS containing 10% sucrose prior to either of the following immuno-electron-microscopical methods.

Cryo-semithin-microtomy

Small tissue blocks, pre-treated with 2.2M sucrose as a cryo-protectant, were mounted on the specimenholder of the Ultracut E/FC4 cryo-ultramicrotome (Leica) and cryofixed by plunging into liquid nitrogen (24). After transfer to the cryo-chamber of the ultra-microtome (at −90 °C), light microscopical sections of 0.5 µm thick were cut and incubated with a saturating concentration of mAb SER-4, stained for immunoperoxidase with the vectastain ABC kit (Vector Laboratories) and counterstained with Methyl Green.

Cryo-ultrathin-microtomy and immunogold labelling

Prefixation and cryoprotection were performed as described above. Ultrathin cryosections of about 80 nm were cut at −100 °C with a diamond knife and immunolabelled according to Tokyuasu (25, 26). Prior to labelling the sections were rinsed 3 times in 0.1% (w/v) glycine in 0.1 M PBS in order to inactivate aldehyde groups. Dilutions of antibodies and immunogold complexes were made in 0.1M PBS containing 0.1% (w/v) acetylated BSA (Aurion Wageningen) in order to prevent background binding of gold conjugates by hydrophobic interaction (27–29). The final working concentration was

determined from dilution series until background labelling over nuclei was below 1% of the total label. Heavy metal staining was performed with 4% (w/v) uranyl acetate (pH 7.0) in 0.3M oxalic acid for 5 minutes and subsequently the grids were placed on a drop of 1.2% (w/v) low viscosity methyl cellulose with 4% uranyl acetate (pH 4.0). This methyl cellulose mixture was removed by carefully blotting the grids on filter paper, yielding a final interference collodion of dried film, in order to prevent the collapsing of sections upon air drying. Control incubation was performed by omitting the primary antibody or labelling with irrelevant antibody and irrelevant polyclonal rabbit serum.

Freeze substitution for low temperature resin and immunogold labelling

After prefixation in PLP fixative as described above, cryoprotection was performed using increasing concentrations of glycerol (10% and 20% during 30 minutes each and 30% during 60 minutes). The tissue blocks were sandwiched with a pair of tweezers between a small piece of plastic (Thermanox) and two low mass copper plates and rapidly frozen into liquid propane with the KF80 apparatus (Leica).

Cryo-substitution of the frozen aqueous constituents was done in a cryo-substitution apparatus (CS-auto; Leica) (30–35). Immersion of the tissue took place in 0.5% (w/v) uranyl acetate in anhydrous methanol as a fixing agent at −90 °C during 30 h. Subsequently, the temperature was raised with 4 °C per hour up to −45 °C and samples were slowly impregnated in increasing concentrations of Lowicryl HM20. Polymerization took place at −45 °C using an U. V. light source attached to the CS auto apparatus. Further polymerization was allowed at room temperature during the next 1 to 3 days. Ultrathin sections cut with a OMU III ultramicrotome (Leica) were rinsed in PBS containing 0.1% (w/v) sodium borohydride and 50 mM glycine and subsequently washed in PBS containing 0.1% (w/v) acetylated BSA. The sections were incubated overnight with the mAb SER-4 at 4 °C, washed and labelled with goat-anti-rat gold probe 10 nm (Aurion Wageningen). As a control, the first antibody was either omitted or replaced by an irrelevant antibody or irrelevant polyclonal rabbit serum. Heavy metal staining was performed with a saturated solution of aqueous uranyl acetate for 5 minutes and a saturated solution of lead citrate for 20 seconds.

Pre-embedding immunocytochemistry

Prefixation was performed with PLP fixative as described above. After washing in 0.1M PBS the tissue was incubated with increasing concentrations of sucrose (10, 15, 20%) in 0.1M PBS and snap-frozen at −20 °C. Cryostat sections of 15–20 μm were cut, collected on non-coated slides and air-dried. Immunostaining was performed according Nakane (10, 23, 36, 37). Control preparations were stained with 25-fold excess of unlabelled SER-4 IgG. Thereafter, the sections were post fixed in 1% OsO4 for 2 h, dehydrated in graded series of ethanol and processed through propylene oxide. A Beem capsule filled with prepolymerised embedding resin epon-araldite was put on top of the section with a drop of resin. After polymerisation for 48 h at 56 °C, the glass slide was broken from the capsule by placing the slide into liquid nitrogen. Thereby, leaving the sections sticking to the capsule. Ultrathin sections of this capsule were cut with an OMU III ultramicrotome and contrasted with uranyl acetate and lead citrate.

All sections were examined by a Philips 301 transmission electron microscope at 80 KV.

Results

Previous light microscopic studies have demonstrated that sialoadhesin is selectively expressed by subpopulations of macrophages in lymphoid tissues, including marginal metallophilic macrophages in the spleen and sucapsular sinus macrophages and medullar macrophages in lymph nodes (10–12). In the bone marrow sialoadhesin is expressed on resident bone marrow macrophages and pre-embedding ultrastructural studies have demonstrated that sialoadhesin is concentrated in areas of contact with surrounding haemopoietic cells (20).

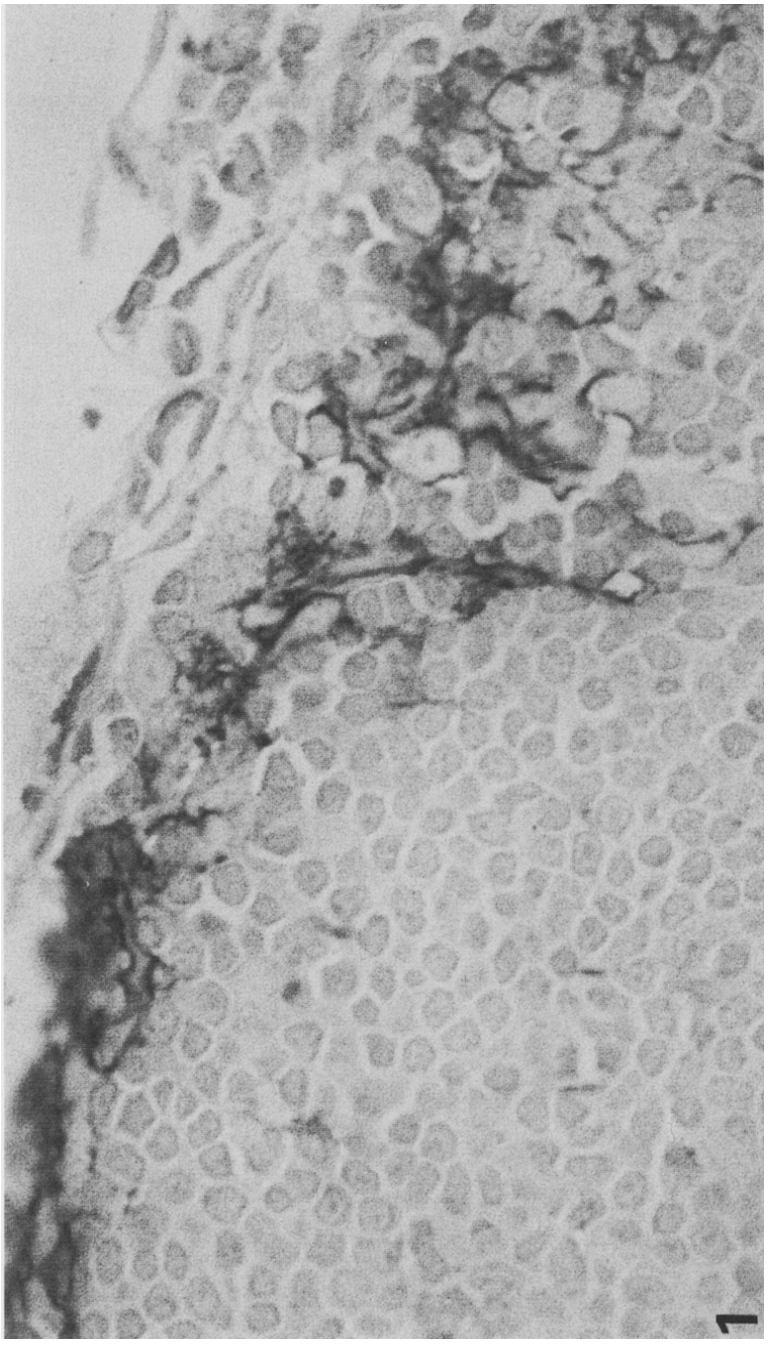


Figure 1. Light microscopical overview of a semithin cryosection (0.5 μm thick) of a mouse poplitial lymph node incubated with mAb SER-4 and stained for immuno-peroxidase. Label is abundantly present on the plasma membrane of macrophages lining the subcapsular sinus. Original magnification: 800×.

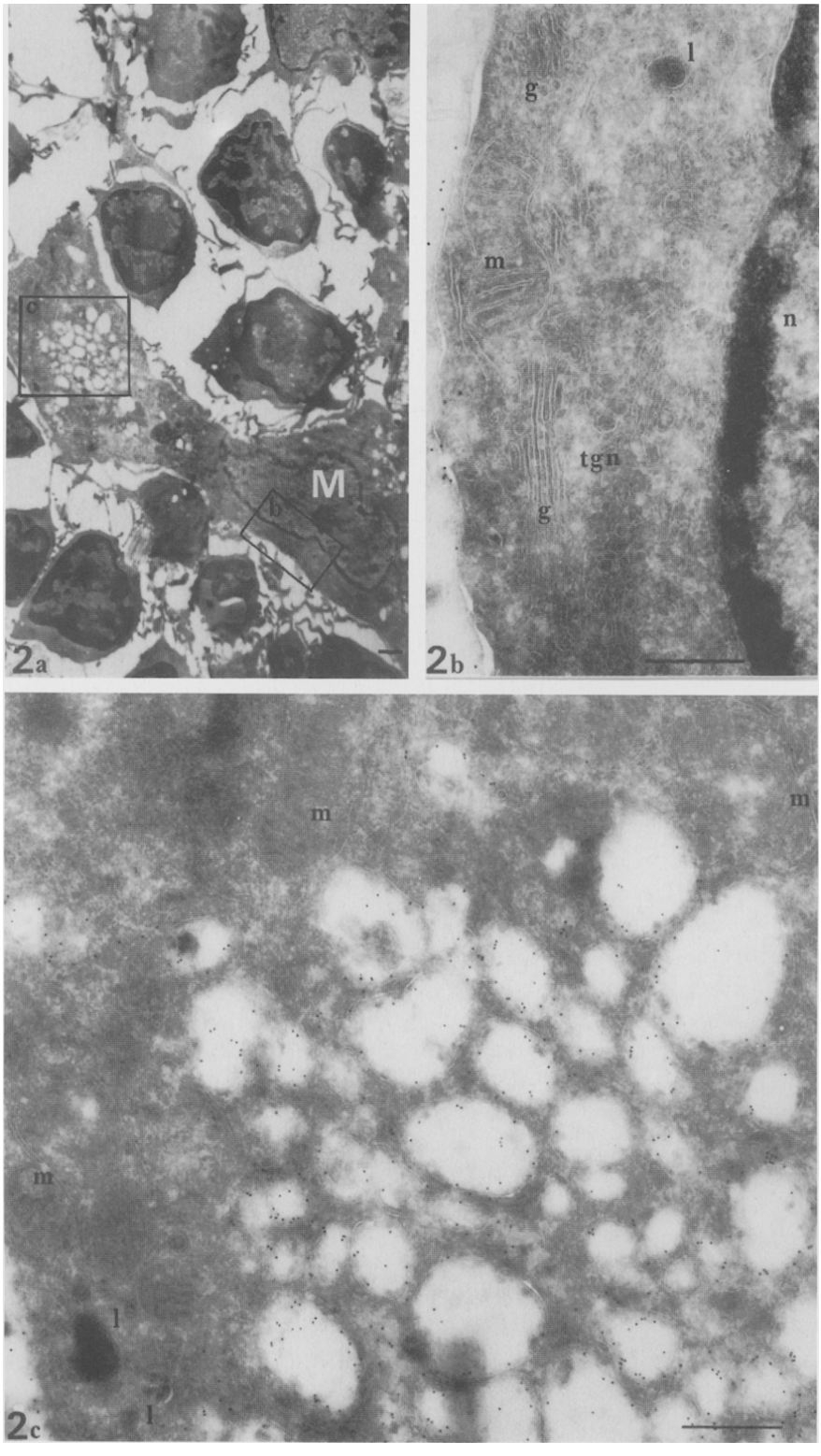
We have investigated the precise intra- and extracellular localisation of sialoadhesin in rodent peripheral lymphoid tissues Figure 1 shows immunocytochemistry using the SER-4 mAb in semithin cryosections of mouse poplitial lymph node. A clear staining was observed of the macrophages lining the subcapsular sinus. The predominant labelling of the cell surface suggests that most sialoadhesin is localised on the plasma membrane. In order to investigate the subcellular distribution of sialoadhesin in more detail several different methods for ultrastructural localisation were performed.

The first technique used was the cryo-ultrathin microtomy method, followed by immunogold labelling. This method is a very reliable way to retain antigenicity and provides good morphology at the cellular level.

At the ultrastructural level we detected label by means of irregular distributed gold particles on the extracellular part of the plasma membrane of subcapsular sinus macrophages (Fig. 2b). In addition, labelling was observed in cytoplasmic vesicles, probably related to the plasma membrane (Fig. 2c). These vesicles have a clear double membrane and the gold label was predominantly located on the inner surface of these membranes. The homogeneous content of these vesicles indicates that they are not lysosomes. There was no labelling detected in ER, golgi, mitochondria or phagosomes. Parallel staining for other murine macrophage receptors like MOMA-1 and ER-TR9 (21, 22), showed a clear surface staining but failed to show the intracellular labelling in vesicles (not shown). Although this technique allows good subcellular localisation, the topography of tissue is significantly disturbed, which hampers a proper evaluation of sialoadhesin distribution in relationship with its putative role in intercellular contacts (38).

In order to circumvent this problem we used the freeze substitution method followed by low temperature embedding, which gives us both a good preservation of topography as well as a high signal on the plasma membrane and in intracellular structures (39–43). In Figure 3a a low power view is shown from the capsule of a mouse poplitial lymph node. Strikingly the macrophages above the sinus lining cells are negative for sialoadhesin while the macrophages and their extensions beneath the lining cells of the marginal sinus are positive. The overall morphology of the positive and negative cells was similar. Figure 3b, a detail of the extensions of the positive macrophage, shows extensive labelling. This labelling is restricted to the plasma membrane and intracellular vesicles related to the membrane. Figure 4 illustrates the localisation of sialoadhesin in relationship to other cells in the mouse poplitial lymph node. In Figure 4a a low magnification of the capsule is shown. Several details of sialoadhesin positive macrophages, give us a clear view of the relationship with adjacent cells like lymphocytes and reticulum cells. Figure 4b shows a detail of the contact sites of two macrophages. Label is located on both the plasma membranes and on vesicles related to the membrane. In Figure 4c it is clearly shown that the invagination of a lymphocyte into a sialoadhesin positive macrophage is labelled on the plasma membrane. Figure 4d provides us a detail of the contact sites of a positive macrophage and a reticulum cell.

It is very difficult to make a quantitative analysis of the amount of gold particles on the contact sites between the different adjacent cells like macrophages, lymphocytes and reticulum cells. This electronmicroscopical approach however, makes it possible to visualise the binding sites of sialoadhesin on the plasma membrane. In Figure 5a and 5b we see a transversal and tangential cut, respectively, through the plasma membrane of a macrophage and an adherent lymphocyte. Note the extensive labelling in Figure 5b over that in 5a.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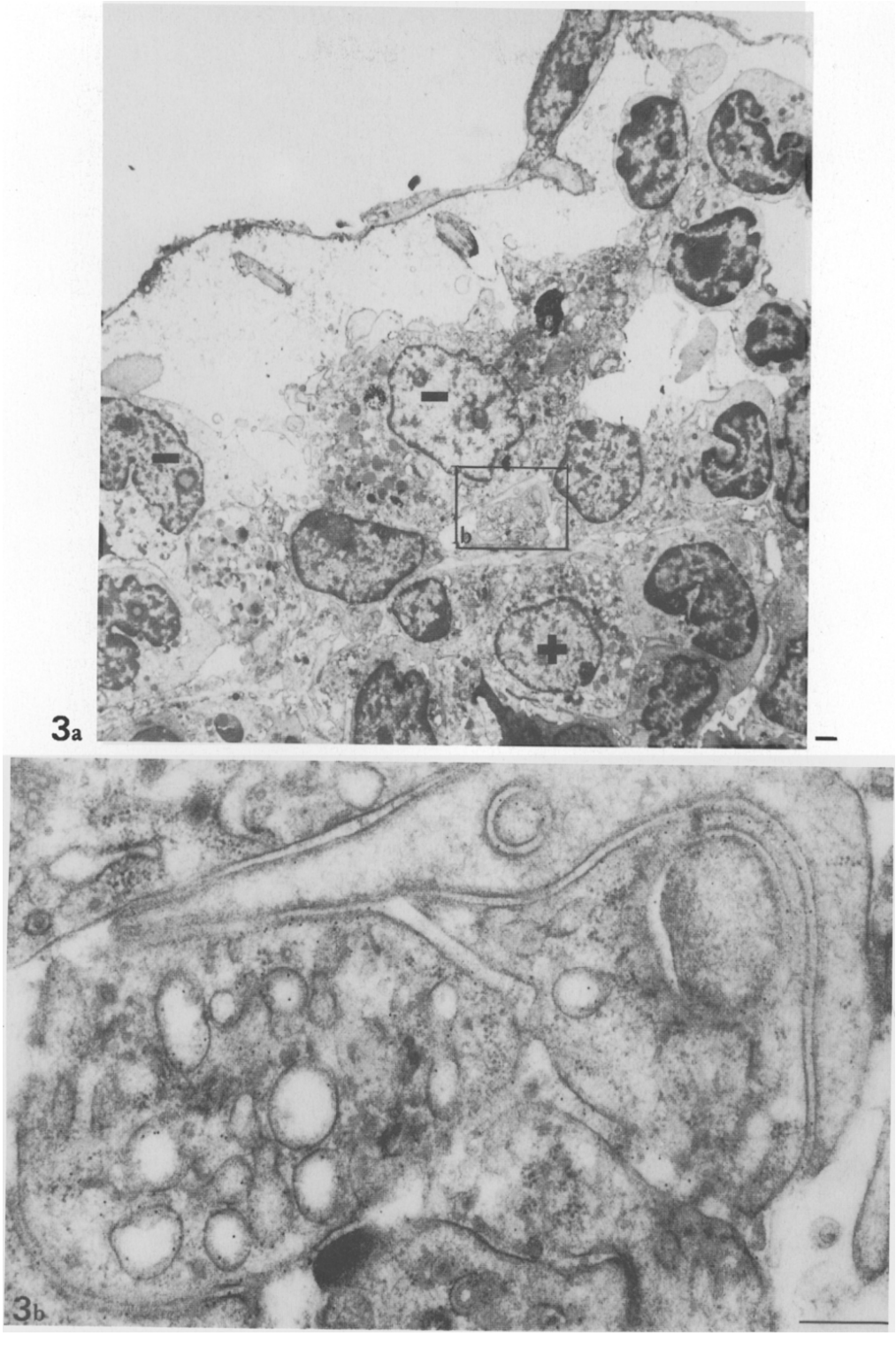

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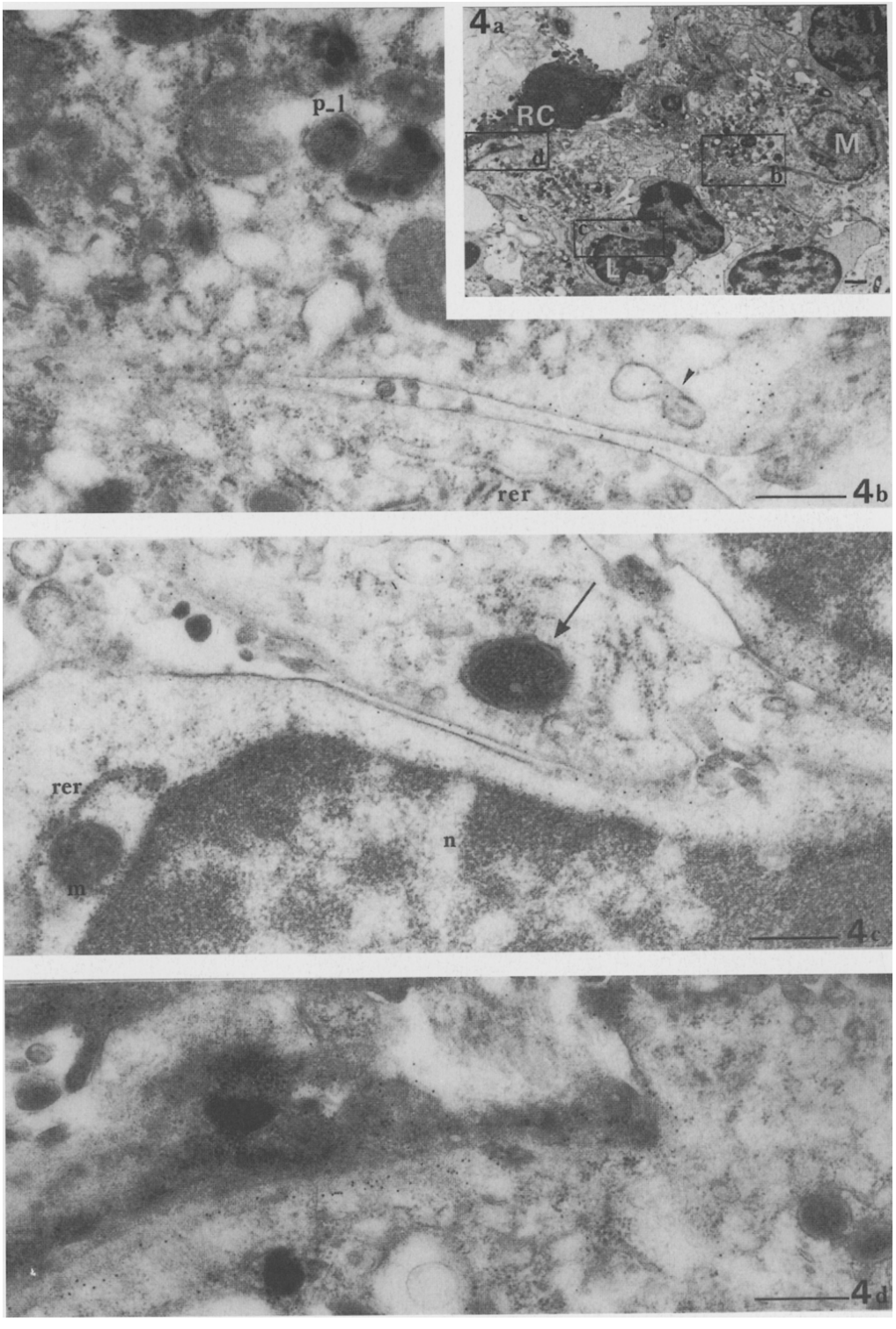
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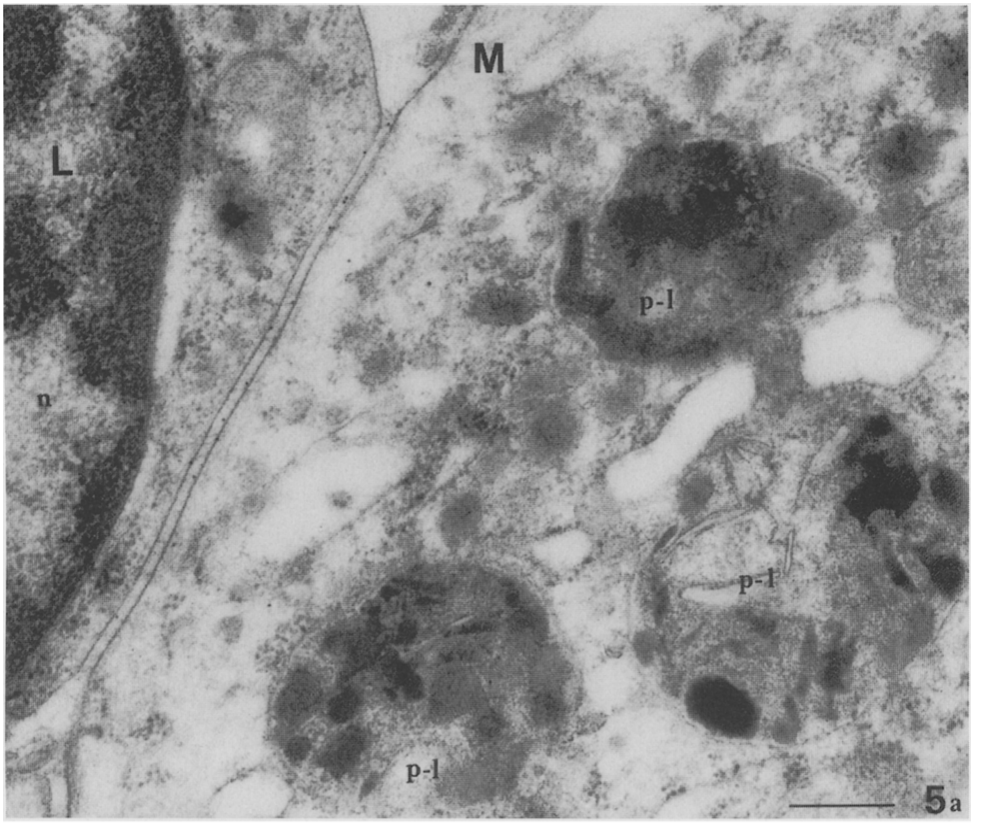
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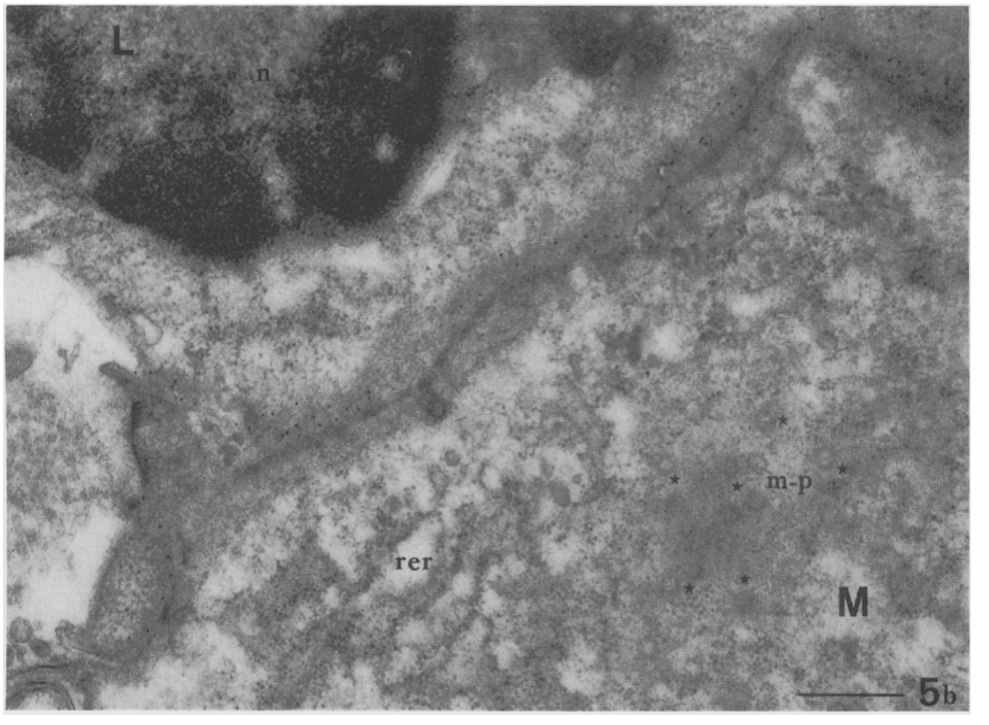
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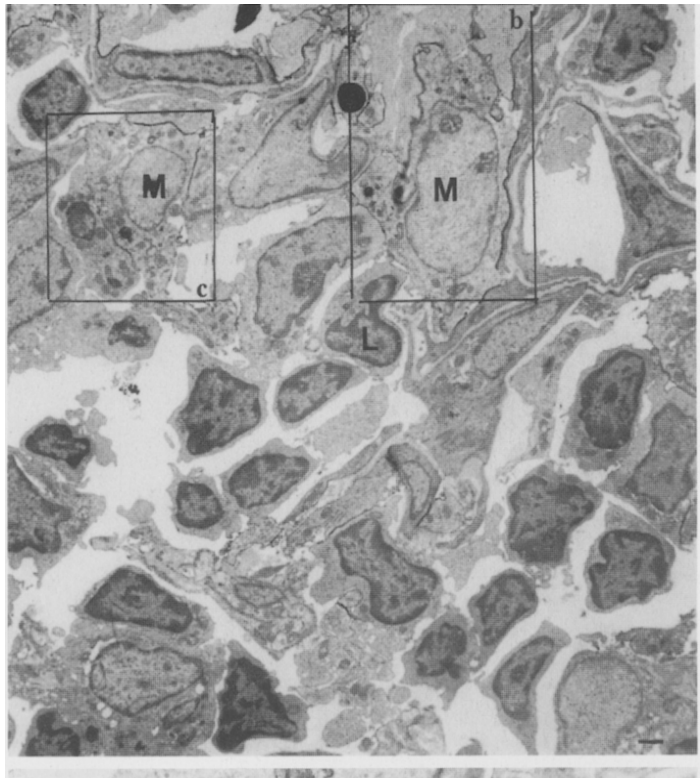

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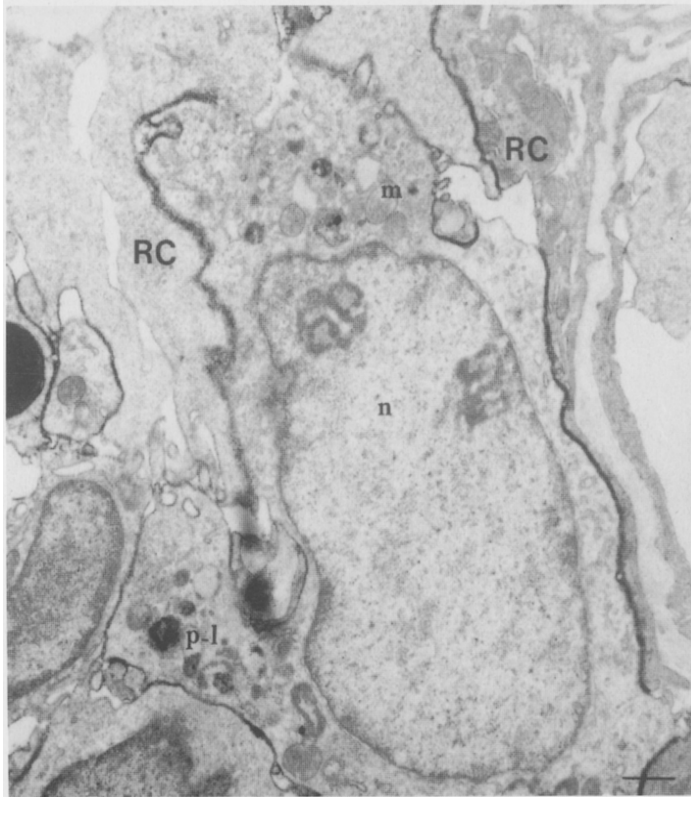

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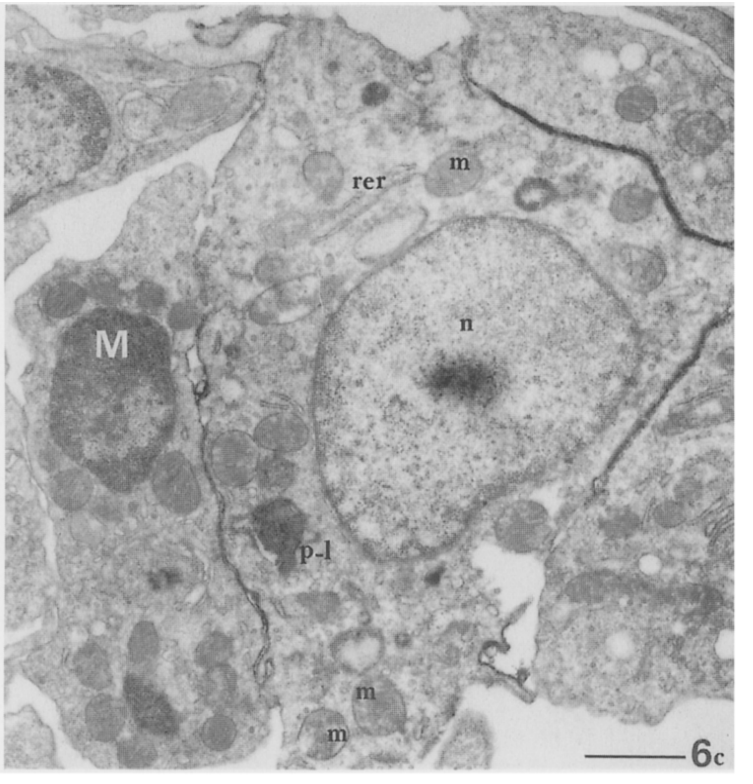
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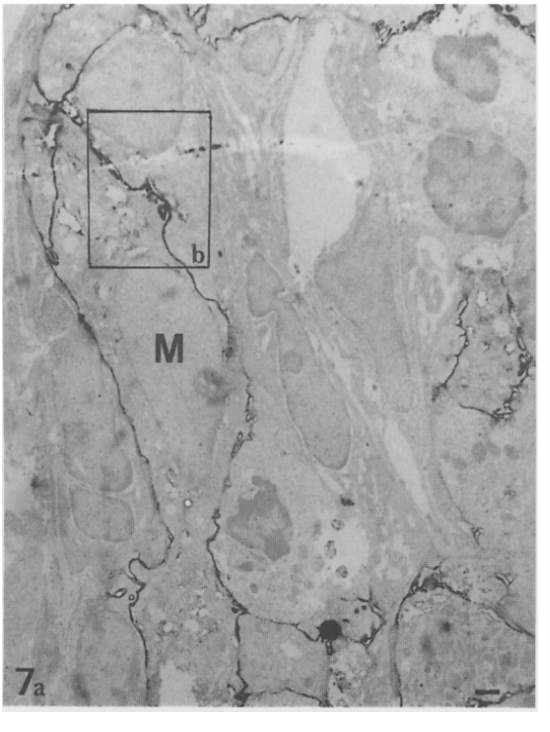
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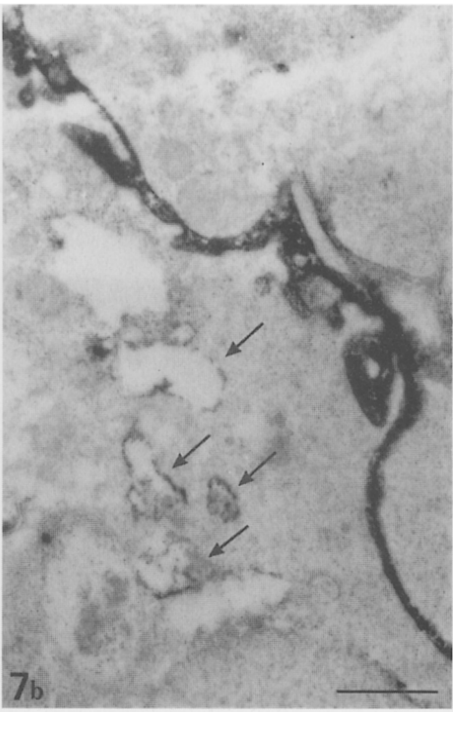
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Figure 2a. Electron microscopical overview of an ultrathin cryosection of a mouse poplitial lymph node (80 nm thick) showing the area of the subcapsular sinus immunolabelled with mAb SER-4 and detected by 10 nm gold particles. The macrophage with inserts b and c is positive for sialoadhesin. Original magnification: 4100×. Bar = 1.2 µm

Figure 2b. Detail of the subcapsular sinus macrophage with positive labelling on the cytoplasma membrane. Original magnification: 54 000×. Bar = 0.37 µm

Figure 2c. Detail of intracellular vesicles with an electron lucent content. The positive labelling is predominantly located on the inner membrane. Original magnification: 41 000×. Bar = 0.5 µm. m = mitochondrium; g = golgi apparatus; tgn = tans golgi network; n = nucleus; l = lysosome.

Figure 3a. Electron microscopical overview of a lowicryl HM20 section of a mouse poplitial lymph node labelled with mAb SER-4. Macrophages on the capsule side of the sinus-lining cells are negative (–) while the macrophages beneath the sinus-lining cells are positive (+) for sialoadhesin. Original magnification: 4100×. Bar = 1.2 µm

Figure 3b. Detail of the extensions of the positive macrophage. Immunogold label is restricted to the plasma membrane and to vesicles closely related to the membrane. Original magnification: 41 000×. Bar = 0.5 µm

Figure 4a. Low magnification of a lowicryl HM20 section showing the capsule of a mouse poplitial, lymph node labelled with mAb SER-4. The macrophages with inserts b–d are positive for sialoadhesin. Original magnification: 4000×. Bar = 1.25 µm

Figure 4b. Detail of the contact site of 2 positive macrophages (M). Label is visible on the plasma membrane and on membrane related vesicles (arrow head). Original magnification: 54 000×. Bar = 0.37 µm

Figure 4c. Invagination of a lymphocyte (L) into an adjacent macrophage (arrow). Note labelling of the membrane. Original magnification: 54 000×. Bar 0.37 µm

Figure 4d. Contact site of a macrophage and a reticulum cell (RC). Positive labelling on the plasma membrane. Original magnification: 54 000×. Bar = 0.37 µm. m = mitochondrium; n = nucleus; rer = rough endoplasmic reticulum; p-l = phago-lysosome

Figure 5a. Transversal section through the plasma membrane of a macrophage and a lymphocyte embedded in lowicryl HM20. Note the positive sialoadhesin labelling restricted to the contact sites. Original magnification: 54 000×. Bar = 0.37 µm. n = nucleus; p-l = phago-lysosomes with multilamellar structures.

Figure 5b. Tangential section through the plasma membrane of a macrophage and a lymphocyte embedded in lowicryl HM20. Note the more extensive labelling on the contact sites over that of Figure 5a. Original magnification: 54 000×. Bar = 0.37 µm. n = nucleus; rer = rough endoplasmic reticulum; m-p (*) = membrane pores.

Figure 6a. Low magnification of the marginal zone in rat spleen. The epon-araldite section was prior to embedding labelled with anti-sialoadhesin mAb ED3 and stained for immunoperoxidase. The macrophages with inserts b and c are positive for sialoadhesin. Original magnification: 4100×. Bar = 1.2 µm

Figure 6b/c. Details of positive macrophages with diaminobenzidine reaction product restricted to the contact sites of adjacent macrophages (M), lymphocytes (L) and reticulum cells (RC). Original magnification: 15 000× (b). Bar = 0.75 µm. 25 000× (c). Bar = 0.8 µm. m = mitochondrium. n = nucleus; rer = rough endoplasmic reticulum; p-l = phago-lysosome.

Figure 7a. Low magnification of the marginal zone in rat spleen. The epon-araldite section was prior to embedding labelled with mAb ED3 and stained for immunoperoxidase. The macrophage with insert b is positive for sialoadhesin. Original magnification: 4100×. Bar = 1.2 µm

Figure 7b. Detail of positive macrophage with abundant reaction product on the plasma membrane as well as in intracelullar vesicles (arrows), related to the plasma-membrane. Original magnification: 50 000×. Bar = 0.4 µm.

Summarizing, we can say that the freeze substitution method gives us both an excellent preservation of topography, a high signal on the plasma membrane and in cytoplasmic vesicles as well as a 'rough' indication of the amount of sialoadhesin on the contact sites of positive cells.

Finally, we performed a pre-embedding immunocytochemistry labelling in order to determine with optimal preservation of topography the ultrastructural localisation of sialoadhesin in rat spleen.

This method was also chosen because, due to the UV-opaque nature of the erythrocytes in spleen, the polymerisation step by use of an UV source in the freeze substitution method is not suitable. This pre-embedding labelling method, however, meets the expectations concerning preservation of topography and retainment of antigenity.

Figure 6a shows a low magnification overview of the marginal zone in rat spleen. Figure 6b and c give in more detail the immunoperoxidase staining restricted to the plasma membrane of the macrophages in close contact with lymphocytes, reticulum cells and other macrophages. It is shown clearly that sialoadhesin is almost exclusively located at the contact zones with these adjacent cells. In addition to the finding that sialoadhesin was not only located at the contact zones with lymphocytes but also on the contact sites with reticulum cells and other macrophages we determined that all marginal zone macrophages and metallophilic macrophages in the rat spleen were positive for sialoadhesin.

In Figure 7a another magnification is shown from the marginal zone. The detail in Figure 7b illustrates the immuno-reactivity in intracellular vesicles that was also observed with the post-embedding techniques described above.

Discussion

Under normal circumstances sialoadhesin is expressed by a subpopulation of macrophages in lymphoid and hemopoietic tissues (9–14). In the marginal zone of rat spleen, all the marginal zone macrophages and metallophilic macrophages that we studied showed a positive staining for sialoadhesin. In the mouse lymph node it was clear that the macrophages in the marginal sinus were negative for sialoadhesin while the macrophages beneath the sinus-lining cells were positive. In contrast, both populations showed labelling with the ER-TR9 marker. Also, no general morphological differences between the sialoadhesin positive and negative subpopulations of macrophages were observed. The difference in sialoadhesin reactivity between these cells may lay in the fact that the negative cells are only just arriving in the marginal sinus via the afferent lymphatic vessel and have not yet interacted with the lymph node microenvironment (44–46). It is clear that in general only (some) populations of tissue macrophages express sialoadhesin, whereas macrophages embedded in tissue cavities, like peritoneal- or alveolar-macrophages, or those behind the blood-brain barrier (microglial cells) or blood-thymus-barrier (thymic macrophages) (9, 10) are generally sialoadhesin negative. A possible explanation is that sialoadhesin expressing tissue macrophages may be directly exposed to the serum component that is apparently able to induce sialoadhesin expression in mouse macrophages *in vitro* (1, 2, 47). Other circulating factors like glucocorticoid hormones and cytokines are also able to induce expression on macrophages (9).

In this study a combination of different pre- and post embedding labelling techniques was used in order to investigate the precise subcellular localisation of this transmembrane adhesion receptor in lymph node and spleen of rodents. The results show that sialoadhesin is expressed on the surface of macrophages particular at areas of intimate contact with lymphocytes, but to our surprise also at apparent contact sites with macrophages and stromal cells (reticulum cells and sinus-lining cells). The concentration of sialoadhesin at contact sites with neighbouring cells supports the *in vitro* evidence that sialoadhesin acts as an adhesion receptor and thus provides (indirect) evidence for such a role in lymphoid tissues.

Our previous work has shown that sialoadhesin can mediate the adhesion of (T and B) lymphocytes and this is clearly supported by the present data. At present the significance of this interaction is not clear. The selective and high expression of sialoadhesin in the splenic marginal zone, an area of intense lymphocyte traffic, suggests a possible role in lymphocyte migration. However, until now we have not been able to demonstrate such a role. Another possibility is that an interaction with sialoadhesin on macrophages with B and/or T cells provides a signal that regulates the activation and differentiation in lymphoid tissues. Again there is no evidence for such a role, but it will be interesting to identify counter-receptors for sialoadhesin on lymphoid cells.

Apart from a role in macrophage-lymphocyte interactions the present results also implicate sialoadhesin in macrophage-macrophage and macrophage-stromal cell interactions. That sialoadhesin can play a role in macrophage-macrophage adhesion has now indeed been demonstrated (VAN DEN BERG et al. unpublished). This suggests that sialoadhesin may play a role in the formation and/or maintenance of the spatial organisation of macrophages in lymphoid tissues and their compartments.

Perhaps more surprisingly was the fact that intracellular vesicles, obvious related to the cytoplasma-membrane, but clearly distinct from golgi, lysosomes and phago-lysosomes, also contained immunoreactivity on the inner membrane of the vesicles. Both post-embedding immunogold labelling techniques showed this phenomenon. However, we feel that this is not necessarily in contradiction with a plasmamembrane origin of the sialoadhesin positive vesicles due to receptor-specific sorting mechanisms.

Although previous studies make it very unlikely that sialoadhesin is involved in phagocytosis, the present data open the possibility that sialoadhesin is involved in the receptor-mediated uptake of e.g. sialylated molecules. However, such a conclusion will clearly require direct evidence.

In conclusion our results demonstrate the selective expression of sialoadhesin on a subpopulation of macrophages in lymphoid tissues and provide evidence for an involvement of sialoadhesin in local cell-cell interactions.

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