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An immunohistochemical study of endopeptidase-24.11 and aminopeptidase N in lymphoid tissues

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SUMMARY

Two cell surface peptidases, endopeptidase-24.11 and aminopeptidase N, thought to be involved in metabolizing regulatory peptides, have been immunohistochemically mapped in pig lymphoid organs using specific monoclonal and polyclonal antibodies. In tonsil, spleen, thymus and Peyer's patches, the endopeptidase-24.11 immunoreactivity exhibited a reticular pattern similar to that previously observed in lymph nodes, where this enzyme is much more abundant. Apart from this location in reticular cells, the only structures seen to express endopeptidase-24.11 were Hassall's corpuscles in the thymus, confirming their reticular cell origin. Aminopeptidase N exhibited a cellular distribution quite distinct from that of the endopeptidase. It was associated with cells scattered throughout the lymphoid organs studied, consistent with its localization in macrophages. In lymph nodes, some fibroblasts buried in trabeculae also stained for aminopeptidase, but this was not observed in spleen and thymus.

INTRODUCTION

Endopeptidase-24.11 (EC 3.4.24.11) and aminopeptidase N (alanyl aminopeptidase, EC 3.4.11.2) are integral membrane proteins found on the plasma membranes of many cell types. They are *ectoenzymes*, with their active sites exposed at the cell surface (for review see Kenny & Maroux, 1982). Endopeptidase-24.11 hydrolyses peptide bonds involving the amino groups of hydrophobic amino acid residues (Kerr & Kenny, 1974; Matsas, Kenny & Turner, 1984). Aminopeptidase N exhibits a broad specificity towards many *N*-terminal residues, with a preference for neutral amino acids, and is more effective with oligopeptides than with dipeptides as substrates (McDonald & Barrett, 1986; Gee & Kenny, 1985). The natural substrates for both enzymes appear to be regulatory peptides rather than proteins, and the topology of the peptidases implies that they have roles in the inactivation of such peptides at cell surfaces.

Endopeptidase-24.11 has been mapped in various tissues, including kidney and intestine (Gee, Matsas & Kenny, 1983), lymph nodes (Bowes & Kenny, 1986) and nervous system (Matsas, Kenny & Turner, 1986). Lymph nodes are, after kidney, the second most abundant source of the enzyme (Gee *et al.*, 1985). Aminopeptidase N is also a membrane enzyme with a wide distribution, including brush borders of kidney, intestine and placenta (Kenny & Maroux, 1982), as well as in lung and liver (Ito, Hiwada & Kikubu, 1980). Histochemical methods have shown an enzyme of this specificity to be present on macrophages from peritoneum and bone marrow, but not on granulocytes or lymphocytes (Wachsmuth & Staber, 1977).

Our previous studies on lymph nodes (Bowes & Kenny, 1986) showed that endopeptidase-24.11 is located in reticular cells. In cryostat sections the immunostaining has a reticular pattern, strongest in the medulla but present in all zones of the node including the follicles. No immunostaining was seen on Ia-positive cells nor on any lymphocytes. The endopeptidase-24.11 content of lymph nodes showed considerable variation from node to node, while the content of other lymphoid organs—spleen, thymus and lingual lymphoid tissue—was at least two orders of magnitude lower than lymph nodes (Gee *et al.*, 1985). There seem to be no comparable studies on aminopeptidase N in lymphoid tissues.

There is increasing evidence to suggest that neuropeptides and other regulatory peptides play an important role in the regulation of the immune system (see e.g. Blalock, Harbour-McMenamin & Smith, 1985). Both endopeptidase-24.11 and aminopeptidase N have been suggested to play an important role in inactivating neuropeptides in the CNS (for review see Turner, Matsas & Kenny, 1985). There is also much interest in the role of peptidases in processing antigens for presentation to T cells but, at least in macrophages, this requires internalization of the antigen rather than hydrolysis at the cell surface (Allen & Unanue, 1984). It is therefore of interest to know the detailed localization of these two membrane peptidases in the immune system. In the present paper we have used immunohistochemical methods to demonstrate their localization in tonsil, spleen,

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thymus and lymph nodes. One striking finding of this survey is that the two peptidases were invariably located in different cell types.

MATERIALS AND METHODS

Animals

Piglets were obtained from the University of Leeds field station. Tissues from slaughter-house pigs were kindly donated by Asda Farm Products, Lofthousegate, West Yorkshire.

Immunocytochemical methods

Lymphoid tissues were obtained either from piglets (2–8 weeks old, killed by exsanguination under anaesthesia with Small Animal Immobilon—0·1 ml per kg, Reckitt and Colman, Hull, North Humberside), or from slaughter-house pigs immediately after death. Tissues were quickly removed, cooled on ice and blocks were prepared, by freezing in isopentane cooled in liquid nitrogen, within 1 hr of removal. The blocks were stored in liquid nitrogen for periods of up to 3 months. Thin frozen sections (5–15 μm) were cut on a Leitz Kryostat, and were taken onto glass cover-slips and air dried. The sections were then fixed either by immersion for 10 min in 4% paraformaldehyde in buffered saline (Gee *et al.*, 1983) or were immersed in acetone at 20° for 5 min. Fixed sections were washed in 50 mM Tris-HCl, 150 mM NaCl, pH 7·4 (Tris-saline) and were treated for 20 min with 0·2% gelatin in Tris-saline. For immunofluorescence, the samples were treated to reduce endogenous fluorescence using 1% sodium borohydride in Tris-saline for 5 min, with several rinses to remove excess borohydride. For immunoperoxidase cytochemistry, endogenous peroxidase was quenched by treatment with 0·01% phenylhydrazine in Tris-saline at 37° for 30 min, followed by several rinses in Tris-saline.

The antibodies used to detect endopeptidase-24.11 were GK7C2, a monoclonal antibody (Gee *et al.*, 1983) in the form of hybridoma culture media, or a rabbit polyclonal antibody, RP109, affinity purified and used at 1 μg/ml (Bowes & Kenny, 1986). Detection of aminopeptidase N involved either monoclonal or polyclonal antibodies. The monoclonal antibody GK8C1 recognized a single 160,000 molecular weight (MW) band by 'Western' blotting and, when linked to Sepharose 4B, purified aminopeptidase N by immunoaffinity chromatography (N. S. Gee and A. J. Kenny, unpublished work). This protein was used to raise a polyclonal antibody (RP133) in a rabbit. An IgG fraction was prepared by a protein A–Sepharose column. It was used at a dilution of 1:2000. The immunofluorescent and immunoperoxidase staining was performed as previously described (Bowes & Kenny, 1986).

For double-labelling experiments, RP109 was biotinylated as follows. After dialysing the IgG fraction (1 mg per ml) against 0·1 M $NaHCO_3$ overnight, *N*-hydroxysuccinimido-biotin (Sigma, Poole, Dorset, Code H1759) 1 mg per ml in dimethyl sulphoxide, was added in a ratio of 1:8 (w/w), and reacted at 20° for 4 hr. The mixture was dialysed against phosphate-buffered saline. The biotinylated antibody was stored in 50% glycerol, 15 mM NaN_3. Biotinylated RP109 (1 μg/ml) and GK8C1 (1:200 ascites fluid) were added together in the first antibody step. Texas Red streptavidin (Amersham International, Amersham, Bucks, RPN1233) was used at a dilution of 1:200 in conjunction with the FITC fluorescent second antibody. Controls were performed by omitting one or other, or both first antibodies and replacing by preimmune serum from the appropriate species.

RESULTS

Endopeptidase-24.11 in secondary lymphoid tissues

The distribution of endopeptidase in pig lymph nodes has been described earlier (Bowes & Kenny, 1986). The immunofluorescence in tonsil (Fig. 1) showed a generally similar appearance. The staining had a reticular pattern, intense in the area surrounding the follicles, and relatively weak in the middle of the germinal centres (Fig. 1a and b). Staining was also pronounced in the areas adjacent to the septa (Fig. 1d). No staining was visible in the endothelial lining of the venules, but their profiles were outlined sharply by the surrounding reticular staining. The squamous epithelium on the buccal surface and the capsule at the opposite surface were unstained. The overall intensity of staining was less than that in most lymph nodes; it was noteworthy that it was necessary to use the polyclonal antibody to obtain sufficient fluorescence for good photography. An immunoradiometric assay for endopeptidase-24.11 (Gee *et al.*, 1985) on two samples of buccal tonsil gave values that were less than those for kidneys or lymph nodes, but comparable with the third most abundant source of the enzyme, intestine.

The spleen contained very low concentrations of endopeptidase-24.11 by immunoradiometric assay (Gee *et al.*, 1985). The monoclonal antibody GK7C2 yielded only very weak staining, and the micrographs in Fig. 2 were obtained after staining with the polyclonal antibody RP109. In Fig. 2a and b, staining of the follicles can be seen: in the former it is fairly symmetrical around the central artery; in the latter it is eccentric. Occasionally, the marginal zone exhibited weak reticular staining (Fig. 2c and d). The red pulp was unstained.

The thymus, like the spleen, was a poor source of the enzyme (Gee *et al.*, 1985) and immunostaining required the use of the polyclonal antibody RP109 (Fig. 3). Throughout the thymic medulla Hassall's corpuscles were usually stained. The staining was typically confined to the outer layers of the cells (Fig. 3g and h). Staining in the rest of the organ was rather variable. Occasionally, staining of the capsule (Fig. 3a, b and c) was observed, again in a typically reticular pattern (Fig. 3c). Even more infrequently, large aggregations of thymocytes were sometimes observed to be encapsulated by connective tissue that was intensely fluorescent (Fig. 3d, e and f) with a reticular appearance penetrating into the peripheral layers of the thymocytes. Where this staining was observed, it could be revealed by either mono- or polyclonal antibodies. Bundles of nerve fibres were also stained positively (cf. Matsas *et al.*, 1986). The medulla of the thymus contained occasional positively stained structures with an apparently complex morphology. One such was followed by serial 5-μm sections, immunoperoxidase stained (Fig. 4). The series (Fig. 4a–i) shows a single solid profile expanding into three hollow structures (Fig. 4c and d), then coalescing into a single tube (Fig. 4g and h) and finally a solid object (Fig. 4i). The total length of this was 85 μm. We suggest that these structures represent Hassall's corpuscles at a later stage in their life-cycle.

The Peyer's patches of the small intestine, and the discrete patches of lymphoid tissue in the large intestine and rectum,

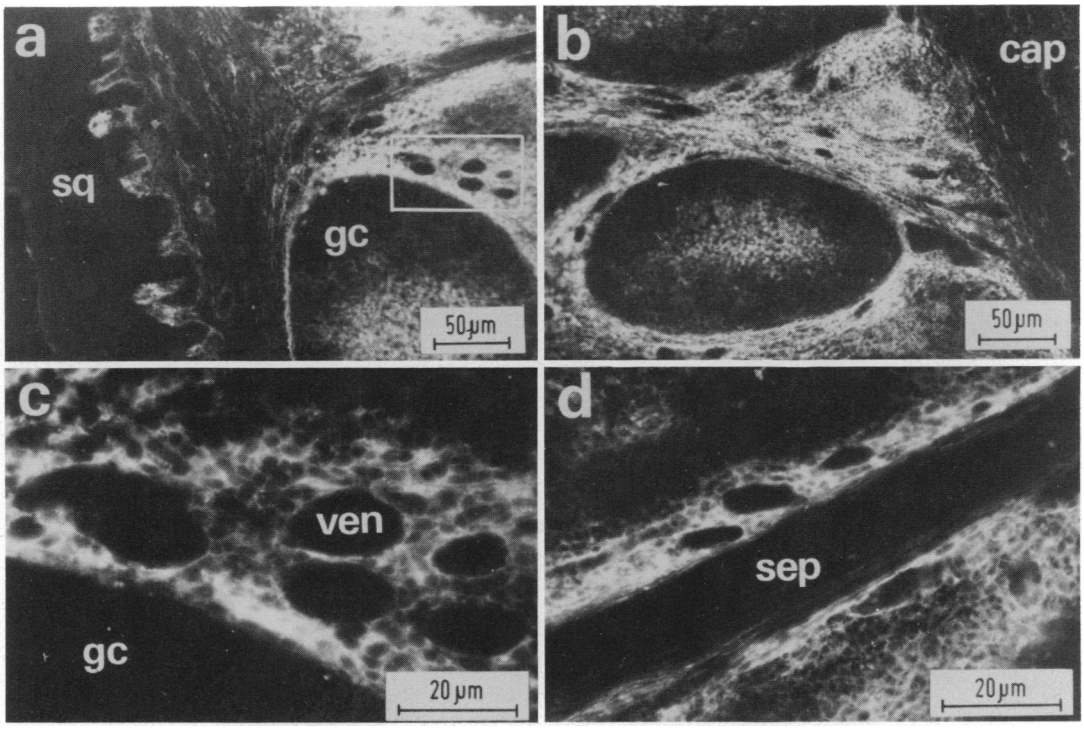

Figure 1. Endopeptidase-24.11 immunofluorescence in tonsil. Sections were 10 μm in thickness, acetone-fixed and stained with RP109 as primary antibody. (a) The buccal surface is visible showing squamous epithelium (sq) and a germinal centre (gc). The intense staining rectangle surrounding the follicle is also shown at higher magnification in (c); the linings of the venules (ven) are not stained. In (b) the deeper region of the tonsil adjacent to the capsule (cap) is shown, together with staining of the germinal centre. In (d) a septum (sep) is visible; it is unstained but there is strong reticular staining immediately adjacent to it.

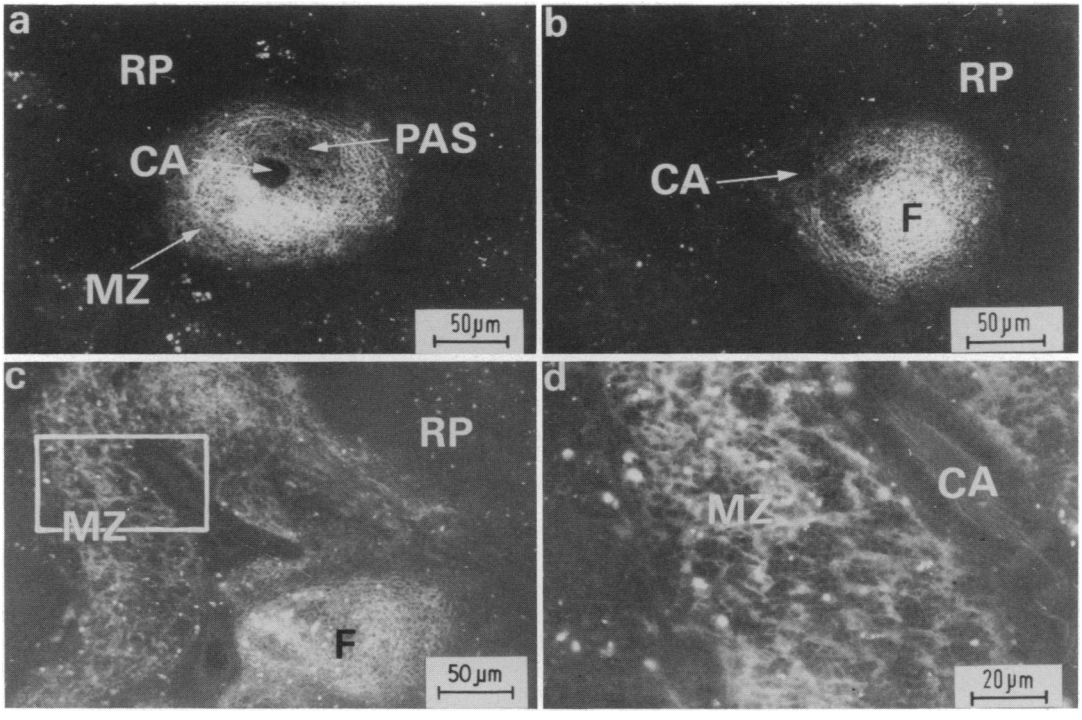

Figure 2. Endopeptidase-24.11 immunofluorescence in spleen. Sections were stained as in the legend to Fig. 1. Two follicles are shown in (a) and (b). CA, central artery; RP, red pulp; F, follicle; PAS, periarteriolar sheath; MZ, marginal zone. In (c) a part of the marginal zone (rectangle) is shown, and at a higher magnification in (d). The bright staining bodies scattered throughout the pulp, seen especially in (c) and (d), are due to autofluorescence.

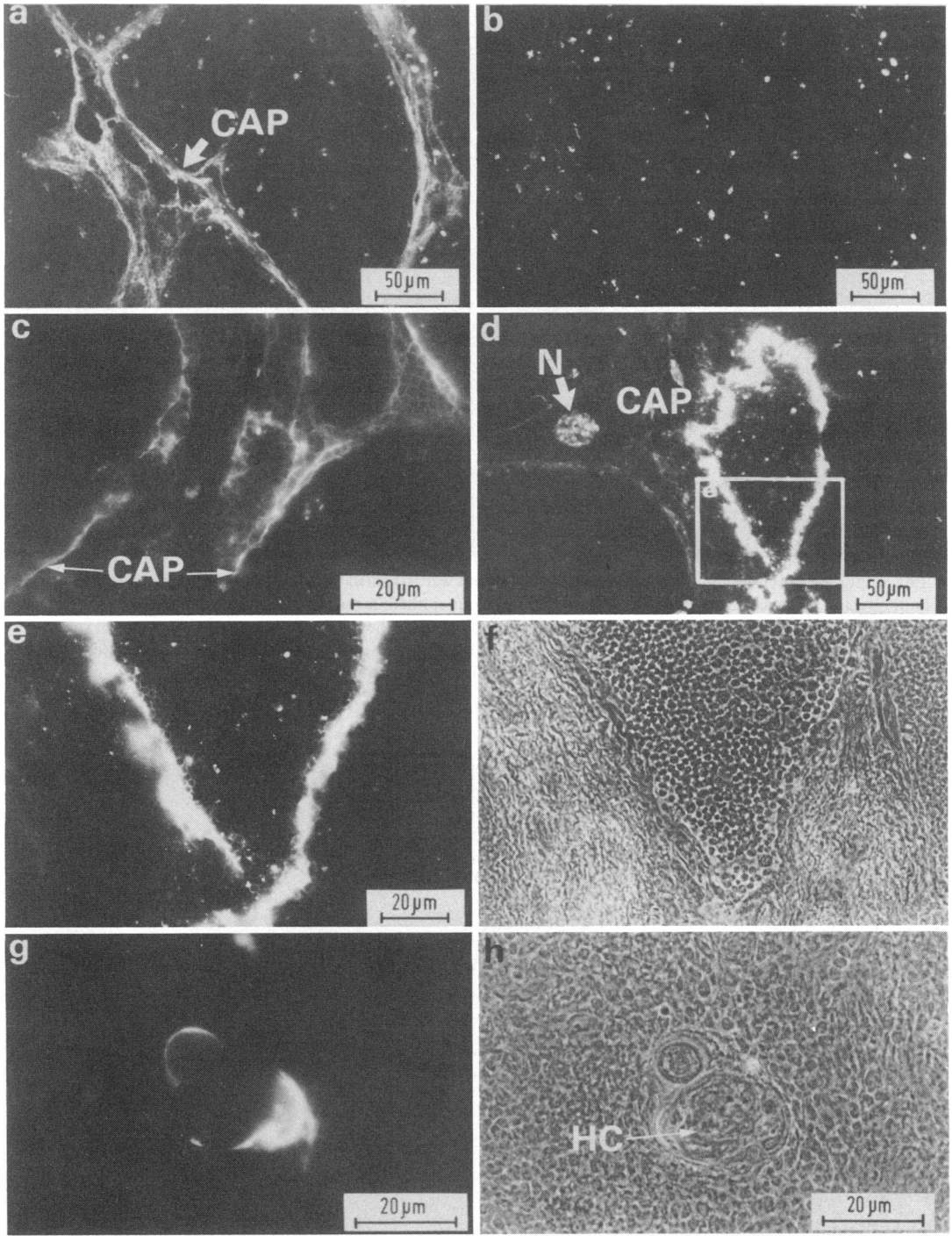

Figure 3. Endopeptidase-24.11 immunofluorescence in thymus. Sections were stained as in the legend to Fig. 1. In (a) an example of staining of the capsule (CAP), rarely seen, is shown; the autofluorescent bodies in the same field are revealed in (b). An area with reticular staining comparable to (a) is shown at higher magnification in (c). In (d) a bundle of nerve fibres (N) is shown and a very intense staining of capsular connective tissue that surrounds a mass of thymocytes. Part of this field (rectangle) is shown at higher magnification in (e) and in phase contrast (f). In (g) and (h) a Hassall's corpuscle is shown; (c) and (e) illustrate the typical reticular pattern of the staining.

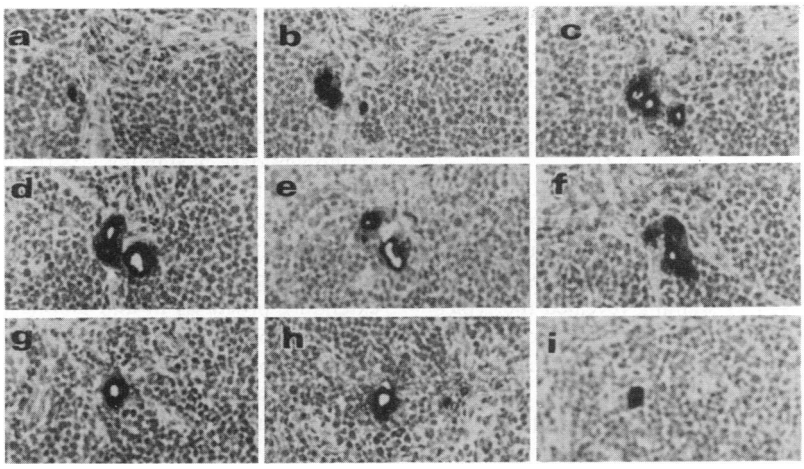

Figure 4. Endopeptidase-24.11 immunoperoxidase staining of thymus. (a)–(i) are 5 μm sections cut serially (intervening 5-μm sections being omitted from the series). The sections were acetone-fixed and were lightly counterstained with Harris' haematoxylin. The structure stained is probably a variant of a Hassall's corpuscle (see text for description).

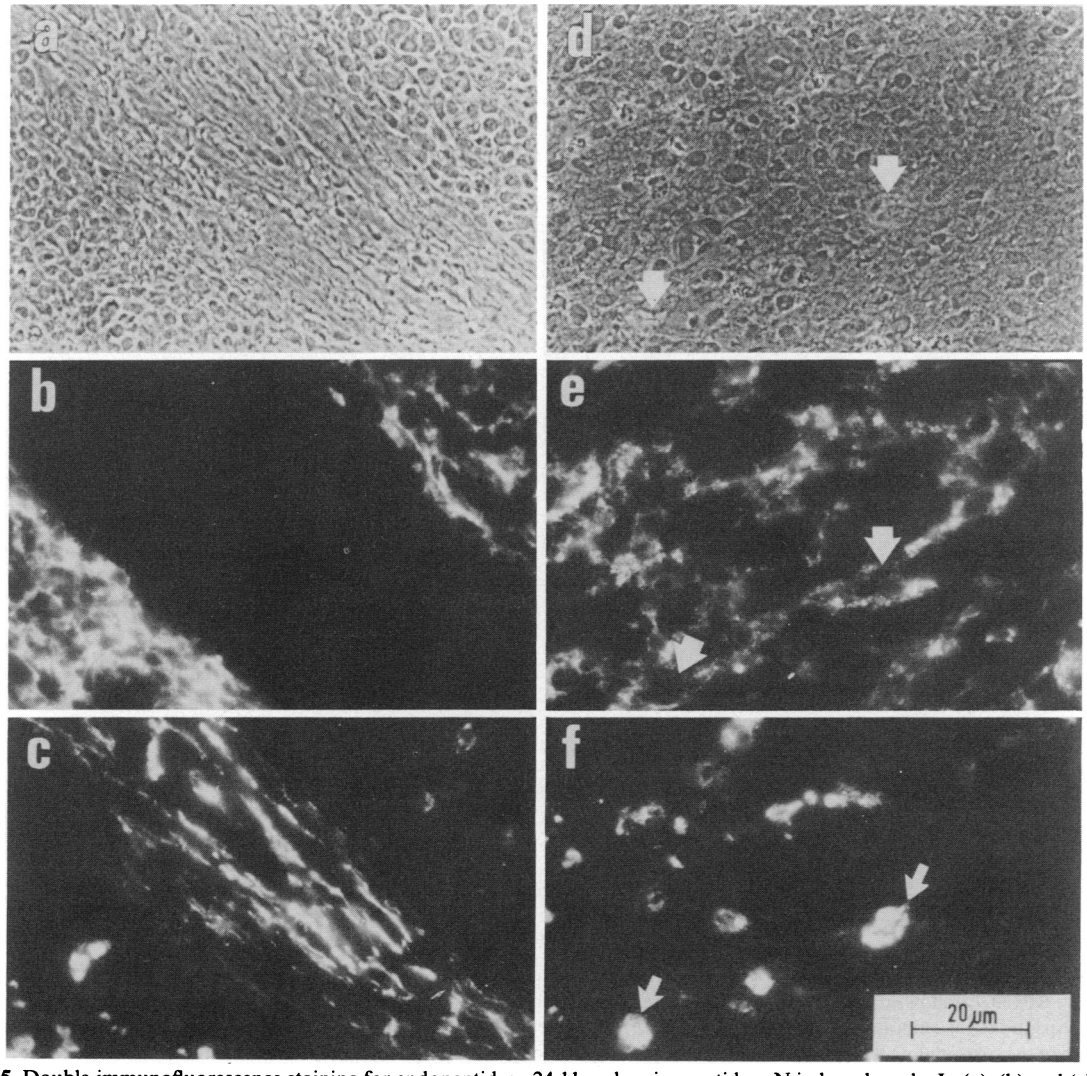

Figure 5. Double-immunofluorescence staining for endopeptidase-24.11 and aminopeptidase N in lymph node. In (a), (b) and (c) one field with a broad trabeculum crossing diagonally downwards from L to R is shown by phase contrast (a), and immunostained for endopeptidase-24.11 (b) and aminopeptidase N (c). A similar series of another field is shown in (d), (e) and (f). While aminopeptidase N was mainly associated with trabecular fibroblasts in (c), it is seen to be also localized in large round cells in (f). Two are indicated by arrows: they are endopeptidase-24.11-negative in (e) and can be distinguished as cells larger than the lymphocytes in (d).

stained in a manner similar to that observed in spleen, i.e. only strong central staining of follicles was observed (detectable with either mono- or polyclonal antibodies; results not shown).

Comparison of the distribution of endopeptidase-24.11 and aminopeptidase N in pig lymphoid tissues

Lymph nodes were stained by the double-antibody protocol (Fig. 5). In Fig. 5a, b and c, a field showing a trabeculum (visible by phase contrast; Fig 5a) was selected. On either side of the trabeculum, endopeptidase-24.11 staining revealed the typical reticular pattern (Fig. 5b). In contrast, the aminopeptidase N staining (Fig. 5c) was predominantly within the trabeculum where elongated fibroblasts were strongly positive. Elsewhere occasional rounded cells were stained. These are better seen in another field (Fig. 5d, e and f). Two cells, staining brightly for aminopeptidase N, are indicated by arrows in Fig. 5f. In the phase contrast view (Fig. 5d) they can be seen to be large, pale cells, and in Fig. 5e the same cells are devoid of any staining for endopeptidase-24.11. The size and scattered distribution of these aminopeptidase N-positive cells suggest that they may be macrophages. We attempted to culture these cells after treating pieces of lymph node with Dispase, deoxyribonuclease I and collagenase (Bowes & Kenny, 1986) but the macrophage-like adherent cells were uniformly negative for aminopeptidase N (result not shown).

The distribution of aminopeptidase N in the thymus, shown in Fig. 6a and b, was strikingly different from that of endopeptidase-24.11. The cells that stained in the thymus were distributed in all areas of the tissue, the staining being more widespread than that of endopeptidase-24.11. A notable feature of the aminopeptidase N staining was the high numbers of positively staining cells found within the thymic medulla, and the comparatively low number of staining cells in the thymic cortex. The cells were, as in the lymph node, much larger than the surrounding lymphocytes, and were frequently seen to be extending processes in and amongst the surrounding thymocytes (Fig. 6b). Their size and scattered distribution suggest that these cells probably belong to the macrophage family. Connective tissue septa throughout the thymus did not stain with the polyclonal anti-aminopeptidase N antibody, unlike the trabeculae of the lymph node.

In the spleen, a similar distribution was seen with cells scattered throughout both the red and white pulp (Fig. 6c and d) but, unlike the staining with endopeptidase-24.11, there was no delineation of follicles or differentiation of various lymphoid compartments. The cells seem to distribute themselves throughout both the white and the red pulp, again suggesting that these cells belong to the macrophage family. The staining of cells within the red pulp was not seen at any time using the endopeptidase-24.11 antibody. Trabeculae within the spleen did not stain for aminopeptidase N.

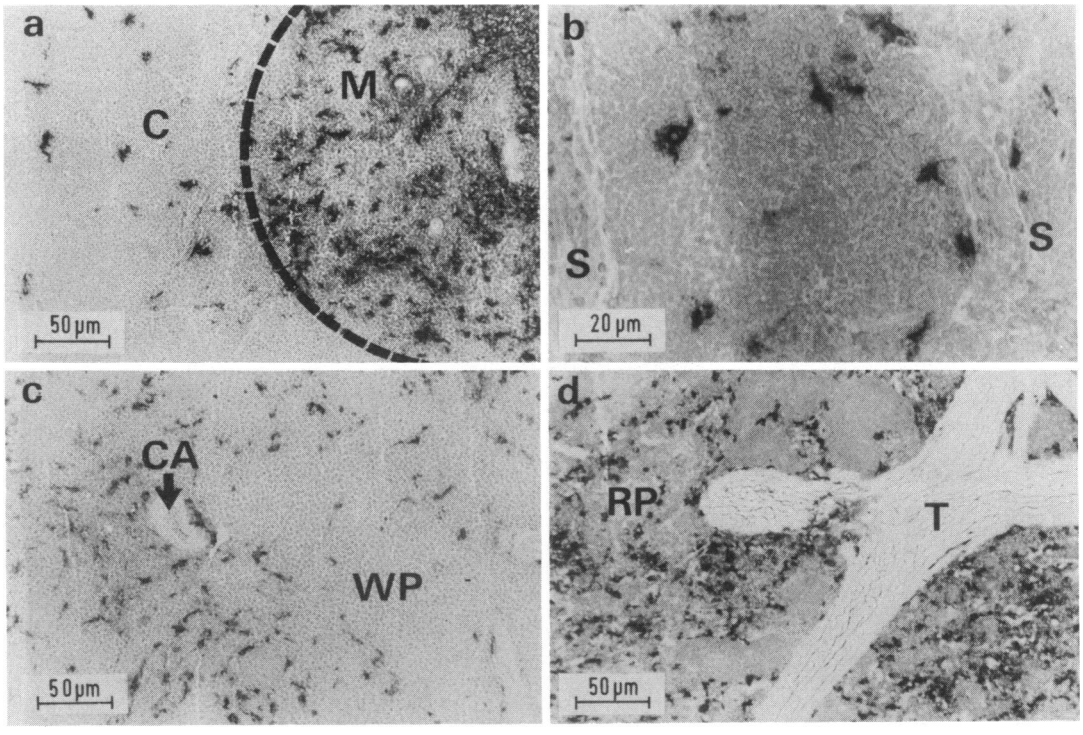

Figure 6. Aminopeptidase N immunoperoxidase staining in thymus and spleen. (a) and (b) depict thymus stained with polyclonal antibody RP133. (a) shows the sparse distribution of positively staining cells in the thymic cortex (c), and the relatively high occurrence of aminopeptidase N staining cells in the medulla (M). (b) is a higher magnification of thymic tissue showing that the cells that stain are larger than the surrounding lymphocytes, and extend processes amongst the lymphocytes that surround them. The connective tissue of the capsule is also seen in this section; it does not stain for aminopeptidase N (S, septa). (c) and (d) show spleen stained with RP133. (c) shows a section of white pulp (WP) with stained cells spread throughout the lymphoid tissue (CA, central arteriole). (d) shows the distribution of stained cells within the red pulp (RP), which is comparable to that of the white pulp. Note the trabeculum (T) which does not stain for aminopeptidase N. The sections were paraformaldehyde-fixed and were lighly counterstained with Harris's haematoxylin.

DISCUSSION

The specificity of the antibodies used in this study had been extensively validated by standard procedures, e.g. immunoblotting, immunoprecipitation and immunopurification of antigen (see Materials and Methods; Bowes & Kenny, 1986; Matsas *et al.*, 1986). However, neither of the polyclonal antibodies was found to cross-react well with other species. The pig immune system is not functionally different from other vertebrates, although pig lymph nodes are anatomically inverted (Binns, 1982; Bowes & Kenny, 1986). Both endopeptidase-24.11 and aminopeptidase N have been studied in other species (rat, mouse, rabbit, human) but comparable studies on lymphoid organs in other species are lacking. In our studies on pig tissues, endopeptidase-24.11 has been identified in many different cell types (Gee *et al.*, 1985). In the nervous system it was localized to the neuropil of the CNS and to Schwann cells in dorsal roots (Matsas *et al.*, 1986). Nerve bundles in many peripheral tissues were stained (M. A. Bowes and A. J. Kenny, unpublished observations) in a manner similar to that shown in Fig. 3d of the thymus.

These immunohistochemical studies have revealed that endopeptidase-24.11 is expressed on the membranes of reticular cells throughout the lymphoid tissues examined, but the intensity of staining varies greatly, being high in lymph nodes and tonsil and lowest in spleen and Peyer's patches, with the thymus being intermediate between the two groups. The only other cell type to show staining for endopeptidase-24.11 was that of Hassall's corpuscles, an observation that would support the view that these structures are derived from reticular cells. The variable content of endopeptidase-24.11 between different lymph nodes and its non-uniform distribution within a node (Bowes & Kenny, 1986) were features that were reflected in the other lymphoid tissues. In contrast, aminopeptidase N was more uniformly distributed, predictably in two cell types—those of the macrophage family, scattered throughout all the organs studied, and some fibroblasts within connective tissue—a feature of the trabeculae of lymph nodes but not seen in spleen, thymus or Peyer's patches. Neither of the peptidases studied was expressed by lymphocytes.

Although tissue macrophages appeared to stain for aminopeptidase N, the adherent cells examined on cover-slips when primary cultures were set up never stained positively for aminopeptidase N. They had the appearance of macrophages and many were Ia-positive (Bowes & Kenny, 1986). This apparently contradictory finding is probably explained by the need to use proteinases in the procedure used to isolate the cells for culture. Aminopeptidase N is very readily released from cell surface membranes, e.g. kidney microvilli, by proteinase treatment (Kenny, 1977), and it is likely that the macrophage surface was stripped of enzyme in this way. After 6–10 days, the cultures became overgrown by fibroblasts, which stained strongly for aminopeptidase N, but no macrophages were seen at this stage.

It is noteworthy that the two peptidases studied were never located on the same cell type. Although the role of neither enzyme has been established in lymphoid tissues, it is likely from what we know of their preference for oligopeptides as substrates (e.g. Matsas *et al.*, 1984) that neuropeptides and other regulatory peptides are their targets at cell surfaces. They may be concerned with the termination of peptidergic signals, and perhaps they play a less specific role as scavengers of peptides, thereby preventing unwanted actions. The two peptidases may well attack different classes of peptide. Many neuropeptides are unavailable to aminopeptidases because the *N*-terminal amino acid residue is blocked (often a pyroglutamyl residue). For these peptides only an endopeptidase can initiate an attack. Others, e.g. the opioid peptides, are potential substrates for an aminopeptidase attack. Considerations of this kind may also relate to the exclusive distributions of these two peptidases.

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