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Immunohistochemical, Morphological and Ultrastructural Resemblance between Dendritic Cells and Folliculo-Stellate Cells in Normal Human and Rat Anterior Pituitaries

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Abstract

Immunolabeling of cryo-sections of human anterior pituitaries obtained at autopsy, and of cryo-sections of freshly prepared rat anterior pituitaries, with a panel of monoclonal antibodies against markers of the monocyte/dendritic cell/macrophage lineage, reveals in both species a characteristic pattern of immunopositive cells, among which many cells with dendritic phenotype are found. Cells characterized by marker expression of MHC-class II determinants and a dendritic morphology are present in both human and rat anterior pituitary. Markers characteristic of dendritic cells such as the L25 antigen and the OX62 antigen were present in anterior pituitaries from human and rat respectively. The population of MHC-class II expressing dendritic cells of the rat anterior pituitary is compared at the ultrastructural level with the folliculo-stellate cell population, which cell type has been previously characterized by its distinctive ultrastructure and immunopositivity for the S100 protein. Using immuno-electron microscopy of rat anterior pituitaries fixed with periodate-lysine-paraformaldehyde, we were able to distinguish non-granulated cells expressing MHC-class II determinants, whereas no MHC-class II expression was found in the granulated endocrine cells. Using double immunolabeling of cryo-sections of these rat AP with 25 nm and 15 nm gold labels, we demonstrated an overlap between the populations of MHC-class II-expressing and S100 protein-expressing cells. Furthermore, MHC-class II-expressing and S100-positive cells showed ultrastructural characteristics that have been previously ascribed to folliculo-stellate cells. At the light microscopical level in the rat AP, a proportion of 10 to 20% of the S100-positive cells was found immunopositive for the MHC-class II marker OX6. In the human AP, S100-positive folliculo-stellate cells and cells expressing the leukocyte common antigen CD45 were found to occupy predominantly different tissue compartments in the human anterior pituitary, namely the epithelial parenchyme cords and perivascular compartments respectively. A proportion of $CD45^+$ cells was found in the parenchyme compartment and, vice versa, indicating an overlap of the tissue compartments in which both cell types occur. However, at the light microscopical level we could not find cells expressing both the S100 and CD45 marker. The present finding of a proportion of S100-positive pituitary cells with ultrastructural and immunohistochemical characteristics of both dendritic cells and folliculo-stellate cells, confirms the suggested heterogeneity of the latter cell group with respect to their ultrastructural phenotype and putative function. The possibility of a myeloid origin of part of the folliculo-stellate cell group in the AP, is discussed and might elucidate some of the discrepancies in the literature concerning the embryological origin of this cell group.

Dendritic cells (DC) with the exception of follicular dendritic cells (1, 2) are defined as a distinct class of cells that are believed to originate from the bone marrow (3). They are found in lymphoid as well as non-lymphoid organs and in peripheral blood and lymph (4, 5, 6). DC have been isolated from many different organs and tissue compartments (7, 8), and were identified *in vitro* by their intrinsic functional properties (7, 9), particularly by their T lymphocyte stimulating properties in the so-called mixed leukocyte reaction (10). To the same class of DC belong the epidermal Langerhans cells (LC) (11) and DC in the liver (12), the gastrointestinal tract (13), the respiratory tract (14) and in various endocrine organs like the thyroid (15) and pancreatic islets (16). The most prominent features of the group of DC are the dendritic morphology and pronounced MHC-class II expression (17). Their outstanding capacity of antigen presentation to lymphocytes makes them more potent accessory cells in the immune response than macrophages (mθs) (18).

The presence of myeloid cells in the normal anterior pituitary

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(AP) has received only limited interest until now. However, a stellate-shaped, non-hormone secreting cell type has long been described as a normal constituent of the anterior pituitary (19), and because of its association with the AP follicular cavities, was called folliculo-stellate (FS) cell (20). These FS cells constitute a network throughout the AP, and are immunopositive for the S100 protein, which is thought to be a neuroectodermal marker (21, 22). Functionally, these FS cells were characterized by their ability to regulate pituitary hormone secretion *in vitro* (23, 24). The similarity between the network of S100 immunopositive FS cells and MHC-class II-expressing DC in the mouse and rat AP, was first described by our research group (25). In the present study, a detailed analysis of the immunophenotype and localization within the AP of cells of the monocyte/DC/mθ lineage and FS cells is elaborated, using immunohistochemistry of the AP of normal humans obtained at autopsy and normal rats, and using immuno-electron microscopy of the AP of normal Wistar rats. Moreover, using double immunolabeling techniques at the light optical (LO) and electron microscopical (EM) level, we investigated the possible overlap between DC and FS cells within the AP.

Results

Pattern of S100 immunostaining in human anterior pituitary (AP)

Immunoperoxidase staining of paraffin sections of human AP with the (polyclonal) S100 antiserum reveals stellate-shaped positive cells scattered throughout the entire gland, corresponding to the description of $S100^+$ FS cells in the rat (22, 21) and human AP (26) (Fig. 1A). $S100^+$ cells were found in clusters or scattered among granulated endocrine cells, and the long slender cytoplasmic processes of these cells extending between granulated cells were also immunopositive (Fig. 1A). In paraffin sections the localization of $S100^+$ cells within the AP was mainly restricted to the epithelial parenchyme cords (27), although a few $S100^+$ cells were found in the perivascular compartment close to capillary lumina. In cryo-sections of human AP immunostained with the S100 antiserum, a clustered distribution of $S100^+$ cells was also found, but here immunoreactivity was predominantly localized in the cell nucleus and perinuclear cytoplasm, which is consistent with the diffusible nature of the S100 protein being a member of the Ca^{2+}-binding protein family (28, 29). In the absence of formaldehyde-based fixatives, S100 immunoreactivity of cryo-sections showed in fact a diffuse, poorly circumscribed staining pattern, possibly due to leakage of the protein out of the cell. Cryo-sections of human AP therefore were not used in double immunolabeling procedures involving the labeling with the S100 antiserum.

In the two non-adult subjects (S92–243 and 236), the AP were entirely negative for the S100 protein, which may be related to the immaturity of the anterior pituitary. Interestingly, Coates & Doniach (30) described that S100 immunoreactivity was absent in anencephalic pituitaries.

Pattern of immunostaining of myeloid cells in human anterior pituitary

A survey of the immunostaining pattern in the human AP for a series of monocyte/DC/mθ-specific markers is given in Table 1. No correlation was found between the post-mortem interval and the intensity of staining with this panel of markers. Numerous stellate-shaped cells bearing MHC-class II determinants (OKIa) (Fig. 2A) were found in cryo-sections of all human AP examined, including the non-adult subjects (cfr. above). Also, cells immunopositive for the leucocyte common antigen or T200 antigen (LCA; CD45) (Fig. 1B) were found in all subjects but one, although in this specimen $CD45^+$ cells were indeed found in the paraffin-embedded material (Table 1). Morphologically, the $CD45^+$ cells were characterized as either of lymphocyte phenotype or of dendritic phenotype, with numerous fine cytoplasmic processes (Fig. 2B). In paraffin sections the $CD45^+$ cells appeared within the lumina of blood vessels, the perivascular compartment and the larger connective spaces (Fig. 1B), but very few $CD45^+$ cells were found in the epithelial parenchyme compartment. The expression of the Langerhans cell marker CD1a and the monocyte marker CD14 was variable in the human APs examined, varying from no or only moderate expression for the CD1a antigen and none or abundant expression for the CD14 antigen. However, a proportion of the $CD14^+$ cells in some heavily stained AP were localized around follicle-like structures (Fig. 2D). Also, dendritic-like cells expressing the L25 antigen (31) were found in high density in some pituitaries (Fig. 2C).

Using a double immunolabeling procedure with a peroxidase staining followed by an APAAP staining method, we found partially overlapping cell populations immunopositive for CD14 and CD45 that also partially expressed the MHC-class II determinant OKIa (Fig. 1D). Moreover, a proportion of the population of $CD45^+$ cells was positive for the CD1a marker (Fig. 1E). However, double immunolabeling of paraffin sections with the S100 and CD45 antibodies revealed that most of the $CD45^+$ cell population was distinct from the $S100^+$ cell population (Fig. 1C). At the light optical level, no clear indication was found of cells expressing both the S100 protein and CD45 marker.

Pattern of S100, macrophage marker and MHC-class II expression in rat anterior pituitary

In cryo-sections of rat AP post-fixed with Zamboni fluid and immunolabeled with the S100 antiserum, a distribution of positive cells was found reminiscent of the $S100^+$ cells in human AP. $S100^+$ cells showed a clustered distribution and were frequently found in association with follicle-like structures. In cryo-sections,

FIG. 1. A, B, C. Paraffin sections of human anterior pituitary immunostained with the polyclonal anti-S100 serum (1A) (×340), with a mAb against the CD45 marker (1B) (×544), and double-immunolabeled with the anti-S100 (red) and anti-CD45 sera (blue) (1C) (×544). $S100^+$ cells are mainly localized within the epithelial parenchyme cords, whereas $CD45^+$ cells are predominantly found in the perivascular and connective spaces.

D, E. Cryo-sections of human anterior pituitary double-labeled with mAbs directed against the CD45 marker (blue) and MHC-class II determinants (red) (1D), and against the CD45 marker (red) and the CD1a marker (blue) (1E) (×857). Note the double immunolabeled cell (arrow) in 1E beside the cells immunolabeled with either the CD45 or CD1a marker (separate red and blue stains). All sections shown are from adult human pituitaries obtained at autopsy. PV: perivascular compartment; EP: epithelial parenchyme compartment (Bars = 20 μm).

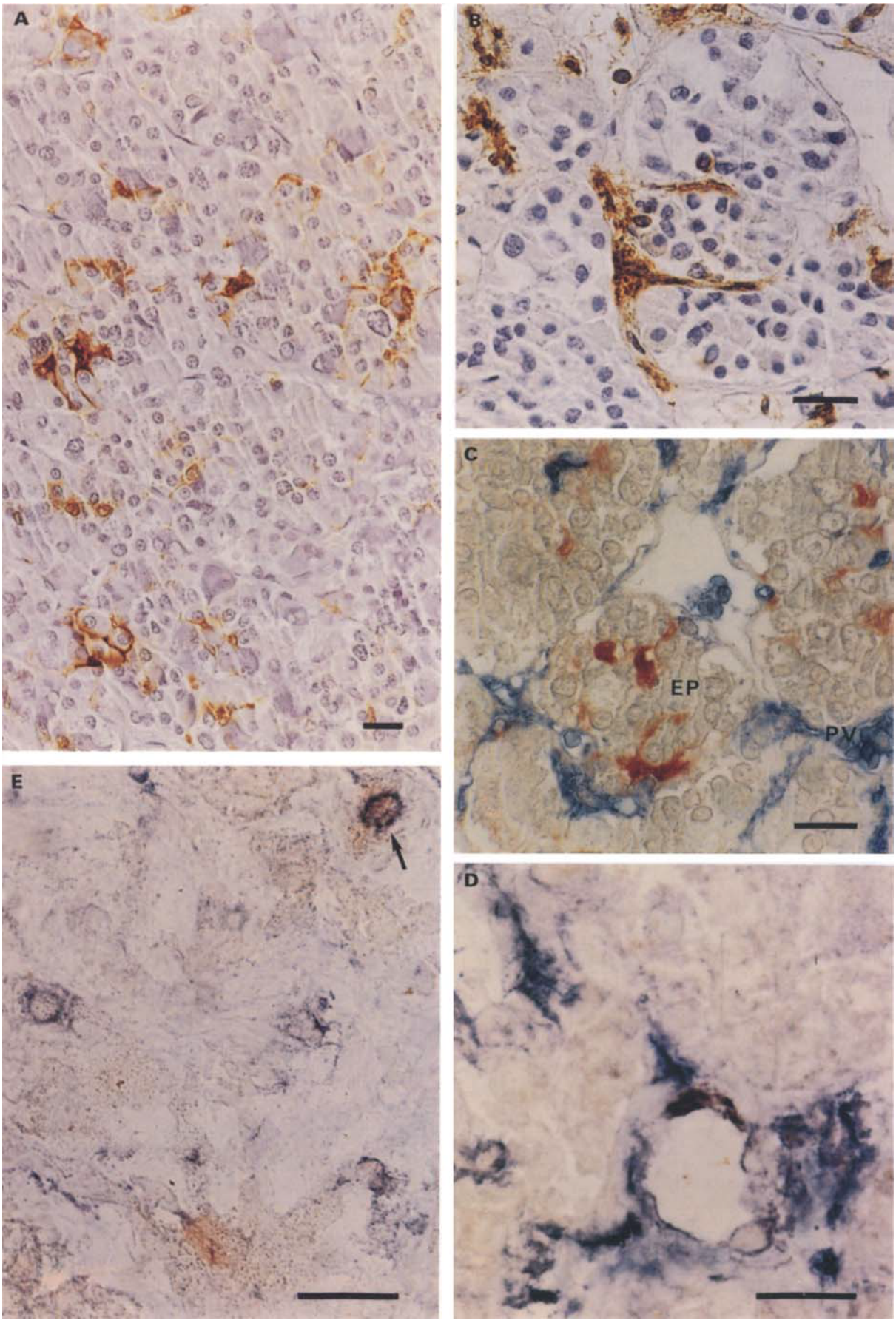
A
B
C
EP
PV
D
E

TABLE 1. Immunostaining Pattern of Human AP with Monocyte/mθ/DC-Specific Markers and Anti-S100 Serum

		Cryo-sections							Paraffin sections	
Patient number	Sex/Age (yr)	OKIa	CD1a	CD14	CD45	RFD1	L25	S100	CD45	S100
S92–13	M/40	++	±	++	++	N	++	++	+	−
S92–108	M/30	++	+	+	++	−	±	++	+	+
S92–119	F/61	±	+	±	+	−	±	++	±	++
S92–123	M/72	+	+	±	+	−	+	+	+	±
S92–128	M/82	+	±	++	++	++	+	++	N	±
S92–142	F/73	++	−	++	++	+	+	+++	N	N
S92–231	M/52	+	±	−	++	+	±	++	++	+
S92–232	M/74	++	±	±	+	+	+	++	+	+
S92–233	M/66	++	±	−	−	++	+	++	+	−
S92–236	M/1 (12 months)	++	+	−	++	±	+	−	+	−
S92–243	F/neonatus (36 weeks)	±	−	−	+	−	−	−	±	−
S92–244	M/55	N	N	N	N	N	N	N	+	+++
S92–246	F/69	++	++	+	++	N	++	++	+	+++

+++ abundant and clear positive staining; ++ numerous cells with distinct positive staining; + few cells with distinct positive staining; ± weak reactivity; − no reactivity; N not determined.

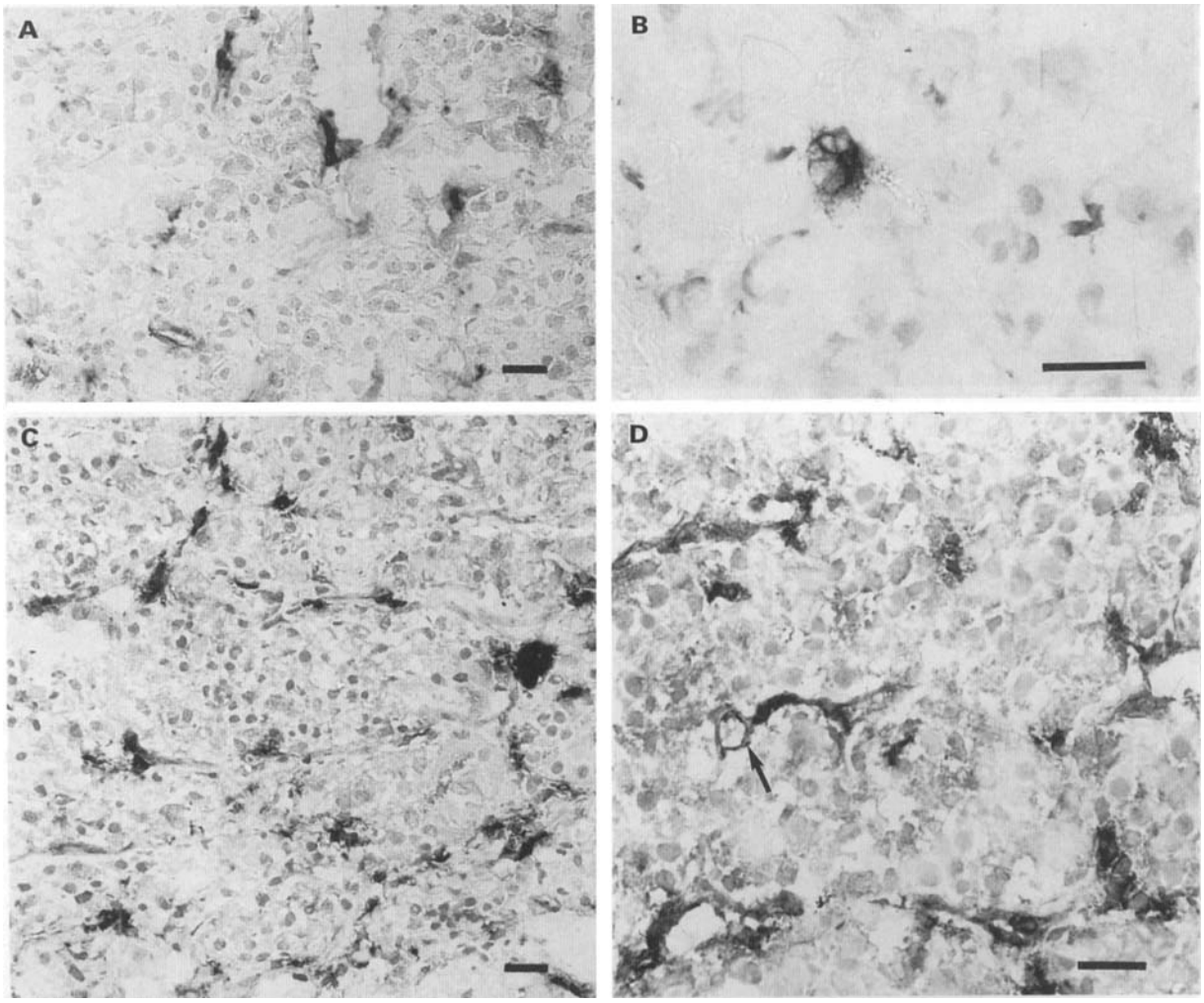

FIG. 2. Cryo-sections of human anterior pituitary immunostained with mAbs directed against MHC-class II determinants (OKIa) (2A) (×272), against the CD45 marker (HLe-1) (2B) (×685), the L25 antigen (TB1–4D5) (2C) (×272) and the CD14 marker (Leu-M3) (2D) (×435). Human pituitaries were obtained at autopsy from adult subjects. Note the $CD45^+$ cell with dendritic-like phenotype in 2B, and the $CD14^+$ cells localized around a follicle-like structure (arrow) in 2D (Bars = 20 μm).

staining was mainly localized in the cell nucleus of these $S100^+$ cells, although a few cytoplasmic processes could be identified.

Immunolabeling of adjacent sections of this rat AP with the MHC-class II marker (OX6) revealed less numerous positive cells (Table 2), but with a similar distribution as the $S100^+$ cells. The ratio of the $OX6^+$ cell number to the $S100^+$ cell number in these adult female rats was about 30% (Table 2). At the light optic level the OX6 mAb stained the cytoplasm, including some (mostly short) processes. Some of the MHC-class II-expressing cells were found adjacent to follicle-like structures (not shown in figure). The recently described DC marker OX62 (33) was sparsely present in the rat AP (Fig. 3A). With regard to the mθ-markers ED1, 2, 3, 7, 8, 9 (34, 35) in normal Wistar AP, only very few immunopositive cells were found. Small numbers of cells weakly positive for the ED1, ED2 (Fig. 3B) and ED7 markers were found, but immunoreactivity with the ED3, ED8 and ED9 markers was absent. A quantitative comparison of the densities of MHC-class II positive, $S100^+$, $OX62^+$, and $ED1^+$ and $ED2^+$ cells is given in Table 2. Using a double immunolabeling of cryo-sections of rat AP after PLP fixation, according to the fixation procedure used for immuno-electron microscopy (see below), we found a clear overlap between the populations of $S100^+$ and $OX6^+$ cells (Fig. 4). About 10 to 20% of the $S100^+$ cells were also immunopositive for the OX6 marker. A number of $OX6^+$ cells was not immunopositive for the S100 marker. A similar pattern of overlap was seen between the populations of $S100^+$ and $OX62^+$ cells (data not shown in Fig.).

Ultrastructure of S100 positive and MHC-class II positive cells in rat anterior pituitary

Ultrathin HM20 sections of rat AP gave excellent topography and morphology of the preselected areas. Immunogold labeling

TABLE 2. Relative Densities of Immunopositive Cell or Nucleus Profiles in Cryo-Sections of Rat Anterior Pituitary*.

Antibodies (source)	Sex/age of Wistar rat	Number of cells or nuclei(1) per mm^2 of section (2)
S100 (Dakopatts; polyclonal)	♀/12–14 weeks	180±7 (3)
OX6 (Seralab; monoclonal)	♀/15 weeks	52±12 (3)
OX62 (Seralab; monoclonal)	♀/15 weeks	18±3 (3)
ED1 (Dr C. Dijkstra; monoclonal)	♀/15 weeks	24±1 (3)
ED2 (Dr C. Dijkstra; monoclonal)	♀/15 weeks	17±7 (3)

*Cryo-sections are post-fixed with Zamboni-fixative (S100 antibody) or acetone (other antibodies) as described in the Materials and methods section.
[1] In the S100 immunostained cryo-sections cell nuclei were counted, because of the marked labeling of these nuclei. In the cryo-sections labeled with monoclonal antibodies, the numbers of immunopositive cells represent the numerical areal densities of whole cell profiles (32).
[2] Mean±SEM (n=number of pituitaries).

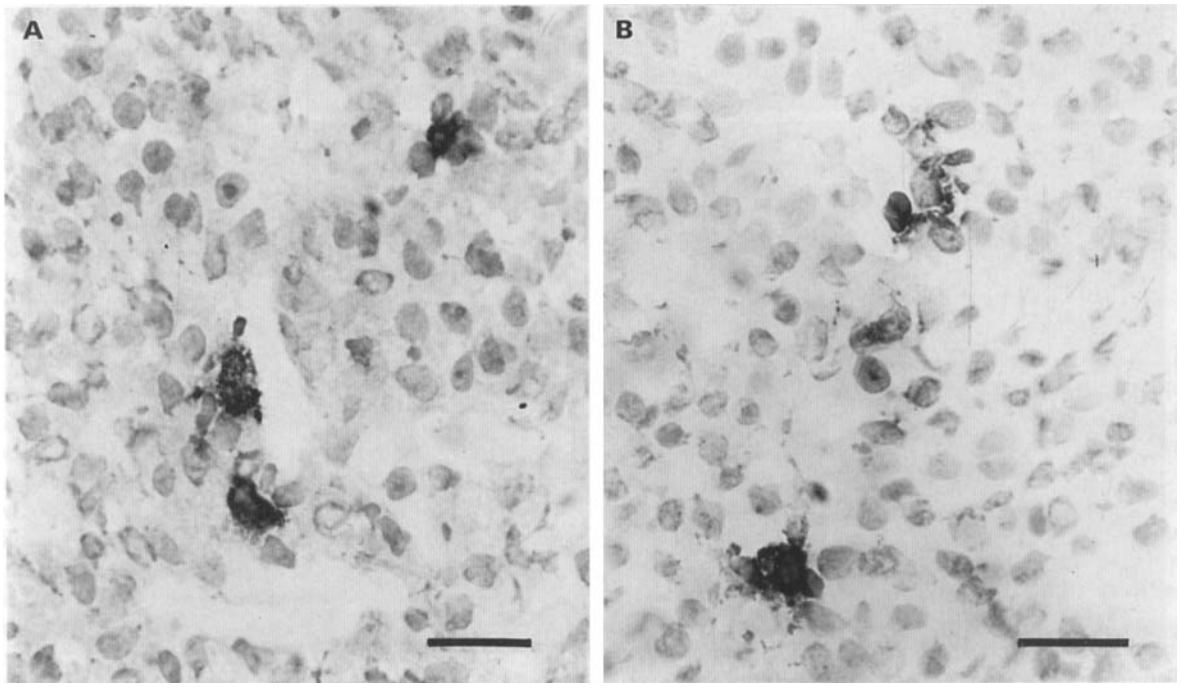

FIG. 3. Cryo-sections of rat anterior pituitary immunostained with the OX62 mAb (3A) (×672) and the ED2 marker (3B) (×672) (Bars=20μm).

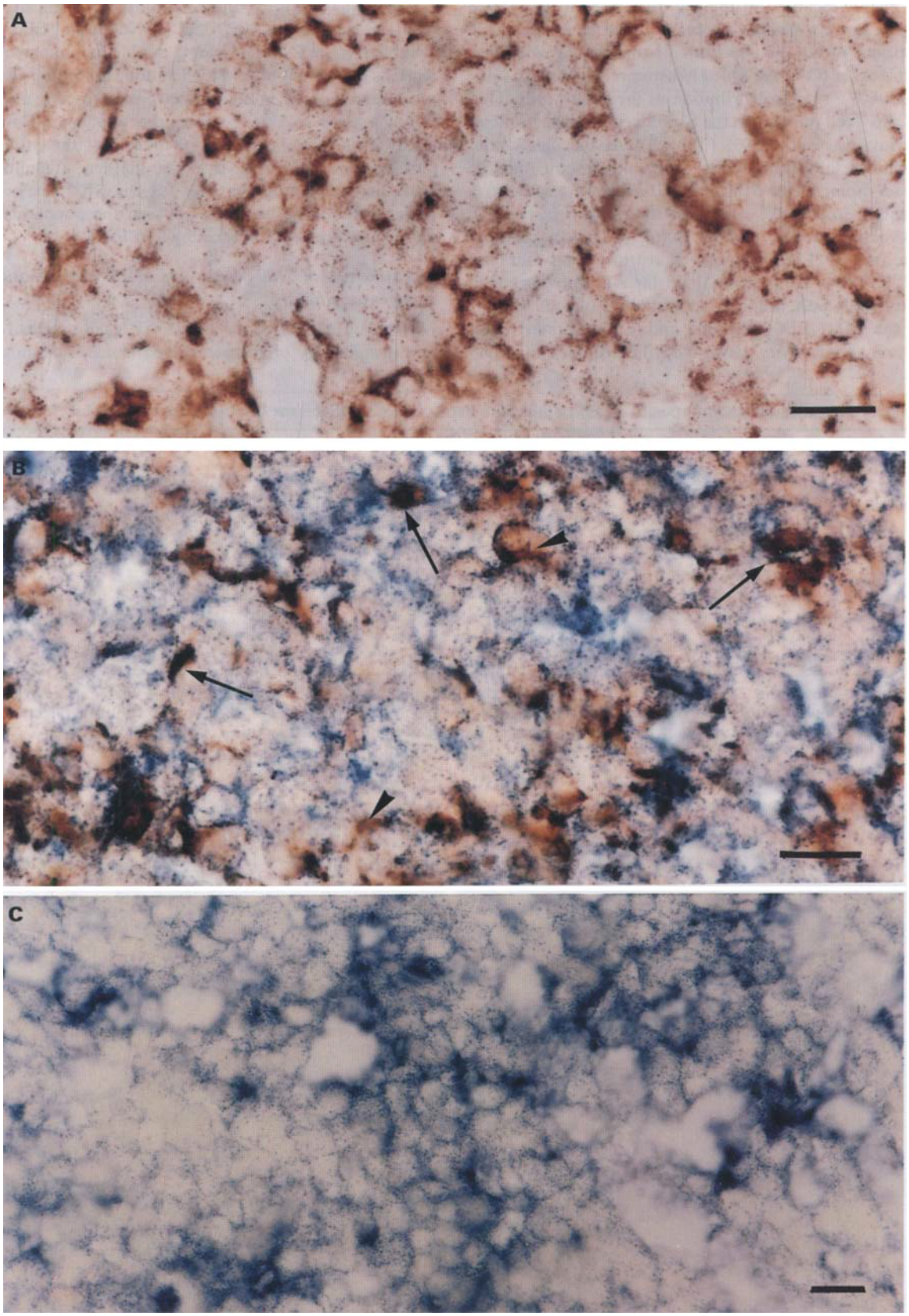

FIG. 4. Cryo-sections of PLP-fixed rat anterior pituitary immunolabeled with the anti-S100 serum (4A) (×672), double-immunolabeled with the anti-S100 serum (red) and OX6 mAb (blue) (4B) (×672), and immunolabeled with the OX6 mAb (4C) (×426). Note the double-immunopositive cells (arrows) and some cells only labeled with the anti-S100 serum (arrowheads) in 4B. Also, cells only labeled with the OX6 mAb are clearly distinguished (blue stain) in 4B. (Bars = 20 μm).

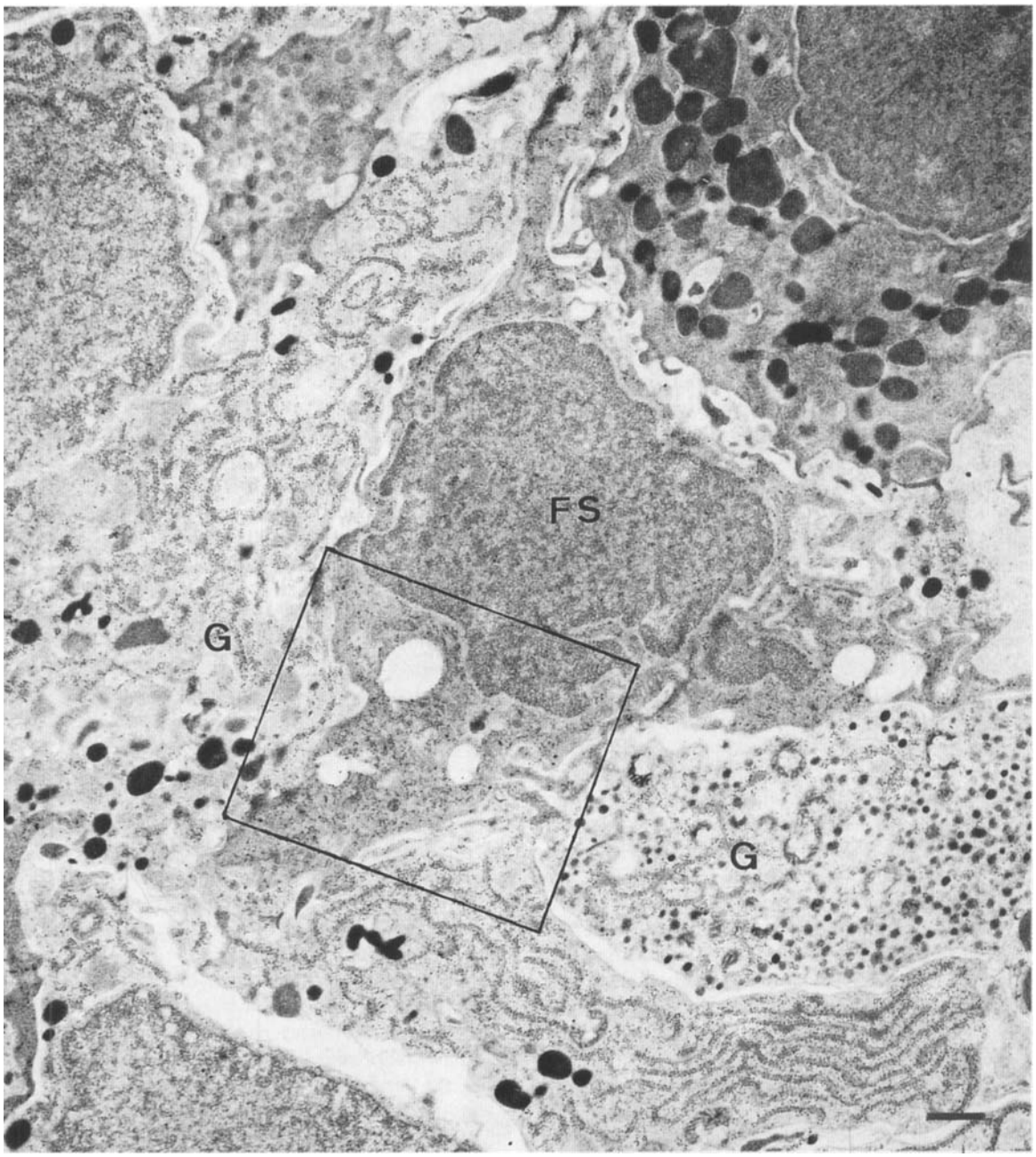

FIG. 5. Ultrathin Lowicryl HM20 section of rat AP giving an overview of a FS cell (FS) surrounded by granulated endocrine cells (G) (×16120; bar=0.5 μm). A high power magnification of the frame is shown in Fig. 6.

with the polyclonal S100 antiserum of HM20 sections (Fig. 5 and 6) revealed cells reminiscent of the folliculo-stellate (FS) cell type as described by Vila-Porcile (20).

Immunogold labeling with the MHC-class II specific mAb OX6 of the HM20 sections, showed fewer labeled cells corresponding with the light-optical staining pattern of $S100^+$ and $OX6^+$ cells (Table 2). Using a double immunogold-labeling procedure with gold particles of 15 nm and 25 nm diameter respectively, we were able to identify FS cells bearing both the S100 protein and OX6 antigen markers (Fig. 5–6). Ultrathin cryosections on the other hand, did not so well preserve the topography of preselected areas, but immunolabeling was more sensitive for both the S100 and the OX6 markers in the cryo-sections than in the HM20 sections (see also ref. 74). Background labeling was absent, for granule-bearing endocrine cells were not labeled with the gold particles.

$S100^+$ cells with stellate, elongated or triangular shape on section were found surrounded by a number of granule-bearing endocrine cells (Fig. 5), indicating a predominantly parenchymal localization. Gold particles labeling the S100 protein were found both in the cell nucleus and cytoplasm (Fig. 6).

Gold particles labeling the OX6 antigen were frequently found associated with the cell membrane, but were also found within intracellular vesicles (36) (Fig. 6). Ultrastructurally, the S100

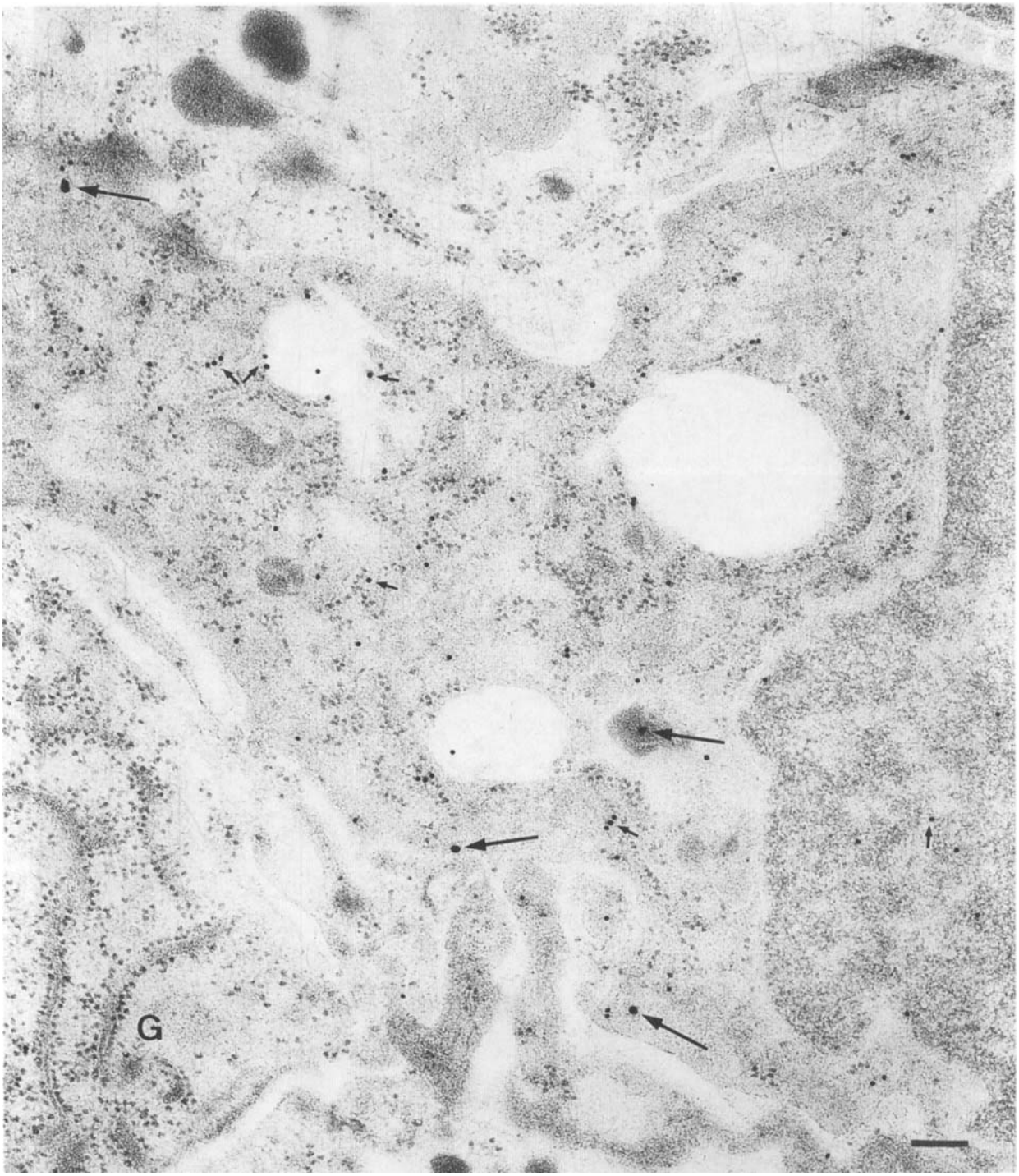

FIG. 6. Ultrathin Lowicryl HM20 section of rat AP showing a detail of Fig. 5: a FS cell is shown at high magnification, labeled for S100 protein (15 nm gold label; shorter arrows) and for MHC-class II determinants (25 nm gold label; longer arrows) (×40660; bar = 0.2 μm). No gold label is found within the granulated endocrine cell (G).

positive and OX6 positive and double-positive cells, displayed characteristics ascribed to both 'classical' FS cells and 'classical' DC, such as a more or less indented nucleus, microvillous projections, many large intracellular vesicles (including lipid droplets and phagolysosomes), elongated or vermiform mitochondria (20) and centrioles. We recently described in more detail several of these ultrastructural characteristics in double immunolabeled ($S100^+$ $OX6^+$) cells in ultrathin cryo-sections of the rat AP (74). Birbeck granulae were not found in the AP cells labeled with either the S100 or OX6 markers. Clusters of FS cells surrounding a follicle, wherein microvillous protrusions are found, were also immunopositive for the S100 and OX6 markers. The

ultrastructural characteristics, immunopositivity for S100 and the topographical localization (24), indicate that this cell type corresponds to the classical FS cells, as described by Vila-Porcile (20).

Discussion

In the present paper the distribution of cells labeled with markers of myeloid cells is described in the AP of human and rat. These data corroborate our previous observations of dendritic-like cells in the rat and mouse pituitary (25). We discuss here how these cells are related to the earlier described FS cells in the AP of various mammals including man (20, 26, 37, 38), because DC and FS cells are very similar at the ultrastructural level. FS cells were described as non-hormone secreting cells with a stellate morphology in the AP (20, 26, 37, 38), that are also immunopositive for the S100 protein which was believed to be a neuroectodermal marker (21, 22). A number of immunohistochemical studies revealed that the S100 protein is also expressed in the skin Langerhans cell (LC) (39), in the interdigitating reticulum cell of the lymph node (40) and in dendritic cells at various locations (41). The importance of these data, together with the present description of dendritic cells in the AP, is highlighted in the following paragraphs. In our group (15, 16, 42) DC are defined as MHC-class II strongly positive cells with dendritic morphology and a limited acid phosphatase activity localized in a juxtanuclear spot (42, 43). Further criteria are a reniform or indented nucleus, and the reactivity to various monoclonal antibodies like in the human RFD1 (44) and the L25 antigen (31) (Fig. 2C and Table 1), and in the rat, the OX62 antigen (33) (Fig. 3A).

The ultrastructural characteristics of most of the MHC-class II-expressing cells in our study are similar to those of DC described in other lymphoid tissues (5). Birbeck granulae (45) the hallmark of LC however, were not found in the pituitary. Part of these MHC-class II positive cells, coincided with the earlier described FS cells (20). We recently described typical vermiform mitochondria, lipid droplets, phagolysosomes and centrioles in the FS-cell like $OX6^+$ cells (74), which characteristics in the AP are classically ascribed to FS cells (20). According to Steinman & Cohn (7) some of these characteristics may also be found in DC (lipid droplets, microvilli, and centrioles), whereas phagolysosomes have been characterized in DC in situ (42). The FS-cell-like cells showed a weak staining with the OX6 antibody, but were frequently labeled with the S100 antibody. FS cell heterogeneity has been described at the light optical (46) and ultrastructural level (47). It is possible that this heterogeneity is reflected in the variable expression of the MHC-class II determinants.

At the light optical level, in cryo-sections of PLP-fixed rat AP, a proportion of about 10–20% of the $S100^+$ FS cells was found immunopositive for the MHC-class II-marker OX6 (Fig. 4). This double immunolabeled cell population probably corresponds at the EM level with the double immunolabeled cells ($S100^+$ $OX6^+$) found in the endocrine parenchyme compartment (Figs. 5 and 6). Comparison of the relative cell numbers found when applying single or double immunolabeling procedures, was suggestive for a relative decrease of S100 antigenicity in the double immunolabeling procedure. This decrease of S100 antigenicity of cryo-sections in the double immunolabeling procedure was seen as a slight reduction of the number of immunopositive cells. This finding is in agreement with the diffusable nature of the S100 protein as observed in cryo-sections of human and rat AP.

Recently, we observed (48) that FS cells and MHC-class II-expressing cells co-localize in the low density fractions of a BSA-sedimentation gradient. Since the numbers of S100 positive cells exceed the numbers of MHC-class II-expressing cells, both *in situ* and in low density BSA-gradient fractions (48) and since the two cell types are ultrastructurally very similar and labeled for both OX6 and S100 (Fig. 4, 6; ref. 74), we conclude that a distinct overlap (about 10–20%) exists between the populations of pituitary FS cells and DC, or, in other words, a subpopulation of the FS cells corresponds with DC.

It was shown that also in the posterior lobe of the human pituitary, pituicytes were found that are immunopositive for MHC class II determinants and/or some macrophage markers (49). However, despite the overlap found between the populations of FS cells and DC in the rat AP, we were not able to demonstrate an overlap between $S100^+$ cells and $CD45^+$ cells in the human AP. CD45 is a marker of the myeloid origin of leukocytes, known as the T200 antigen (50). Using paraffin immunohistochemistry at the LO level, we demonstrated that $S100^+$ cells and $CD45^+$ cells do not match with respect to the compartments in which they are localized, being the parenchymal cords or the perivascular connective tissue spaces, respectively (Fig. 1 A–C). However, a small number of $CD45^+$ cells was also found in the parenchyme compartment. No co-localization was found within the same cell of both the S100 protein and the CD45 marker, which, however, may also be due to the limited resolution of the LO parafin immunohistochemistry. This confirms a previous observation of Vila-Porcile (27), with regard to a 'cellule d'aspect myoïde' or a presumptive myoid cell (27, p. 104) found in the perivascular compartment, but also in the parenchyme compartment. It should be noted that this cell type was described as a distinct cell type, that differed from the FS cells by a number of ultrastructural characteristics, like the relatively dense and indented nucleus and the short and bulbous cytoplasmic protrusions. However, the same so-called 'myoïd' cell also differed from the macrophage cell type described by the same author in the rat AP (27, p. 106). With regard to the mø's of the AP, Vila-Porcile (27) stated that these cells were always found in the proximity of the blood vessels. Moreover we found small numbers of cells with the macrophage phenotype immunopositive for the ED1, ED2 and ED7 markers in the rat AP (34, 35). This indicates that beside the DC population also a $m\theta$ population resides in the rat AP.

Our finding of an $S100^+$ and $OX6^+$ subpopulation of FS cells resembling DC in the rat AP, and the absence of an overlap between the populations of $S100^+$ and $CD45^+$ cells in the human AP, raises some doubt with respect to the embryological origin of the FS cells as reported in the literature. First, the coincidence of S100 and OX6 markers within one cell type suggests that the population of FS cells of the AP might be heterogeneous, as was also described by others (46, 47, 51). Since the S100 protein is also expressed in bone-marrow derived DC, the neuroectoderm, as suggested by Cocchia & Miani (21) and Nakajima *et al.* (22), or the oral ectoderm, as suggested by Gon (52) and Tachibana & Yamashima (51), are probably not the only possible embryological sources of $S100^+$ cells with dendritic phenotype within the AP. The possibility of a myeloid origin of part of the S100 expressing FS cell population in the AP, remains to be elucidated.

With regard to the presence of $m\theta$ markers in the rat AP, we

have shown that the numbers of mθs found were below the numbers of MHC-class II-expressing cells and far below the numbers of $S100^+$ cells (Table 2). Since the markers ED1 and ED7 were reported to partly coincide between mθs and DC (35), and since mouse DC are negative for the mθ marker F4/80 present in the mouse AP (53, 54, 55), we may conclude that the population of pituitary mθs may partly coincide with the DC-like cell population, but only to a very limited extent with the FS cell population. Further research will also be needed to explore the functional relationships between MHC-class II expressing DC and $S100^+$ FS cells of the AP, by studying the functional behavior of subpopulations of these cells in culture.

It is well established that FS cells have an endocrine regulatory function, as was inferred from their ability to regulate pituitary hormone secretion *in vitro* (29). Moreover, recent evidence has shown that S100-containing FS cells contain bioactive and immunoreactive interleukin-6 and are the likely producers of interleukin-6 in the AP (56). On the other hand, the immune regulatory function of DC is indicated by their capacity of antigen presentation to T-lymphocytes (10) and pituitary derived DC are capable of stimulating T cell proliferation in an allogeneic mixed leukocyte reaction assay (74 and unpublished observations). A possible myeloid origin of FSC-DC in the AP also has clinical relevance. Of 13 human pituitaries studied, the absence of S100 expression was revealed in two paediatric cases, one of which had retarded brain development. It is tempting to speculate that the absence of S100 expression in the AP and the absence of certain markers of the DC lineage in the AP, are caused either by a common disturbance or by immaturity. Moreover, the present observation of DC within the AP further confirms the presence of DC in endocrine organs under non-pathological conditions (15, 16, 57).

Materials and methods

Human pituitary tissue

Human pituitaries were obtained at autopsy from 11 adult subjects (8 male and 3 female), 1 female neonatus of 36 weeks old (pathology ref. S92–243) and 1 male child of 1 year old (pathology ref. S92–236). All subjects died from non-endocrine causes, such as coronary insufficiency or cancer. The post-mortem interval was between 6 and 24 h. The age range of the adult subjects was between 30 and 82 years. One of the non-adult cases was diagnosed as a Potter syndrome with lung hypoplasia and renal agenesis (S92–243). The other one was suffering from micro-cephalic development with almost absent pituitary (S92–236).

Human pituitary tissues were divided into 2 parts: 1 part was embedded in Tissue-Tek (Miles Inc., Elkhart, IL, USA) and stored at −80 °C; cryosections of 4 to 6 μm were cut. A second part was fixed in 10% neutral buffered formalin and embedded in paraffin; sections of 5 μm were cut.

Rat pituitary tissue

Adult male and female Wistar rats (3 months old) were prepared for cryo-immunohistochemistry as described previously (25). Male and female rats of 3 weeks up to 3 months of age were studied in parallel in a quantitative light microscopical study of DC development (data presented elsewhere). Pituitaries were embedded in Tissue-Tek and stored at −80 °C. In addition, 6 adult female rat pituitaries were prepared for immuno-electron microscopy. Pituitary tissue was fixed with periodate-lysine-paraformaldehyde (PLP) fixative on ice (4 °C) immediately after dissection according to McLean & Nakane (58).

Paraffin immunohistochemistry

Primary antisera used on formalin fixed and paraffin embedded pituitary tissue were the polyclonal rabbit anti-bovine S100 protein (Dakopatts, Glostrup, Denmark) (diluted 1:1200) and the monoclonal mouse-anti-human leukocyte common antigen (LCA) (CD45; Dakopatts) (diluted 1:12.5). The paraffin sections were deparaffinized with absolute ethanol. Endogenous peroxidase activity was blocked by incubation with 3% methanol in distilled water for 25 min, whereafter the sections were shortly rinsed with tap water. Single immunostaining procedures were performed according to the ABC-method (59) with 3,3′-diamino-benzidine (DAB) (Fluka, Buchs, Switzerland) as staining reagent. The secondary antiserum used was biotinylated goat-anti-mouse and rabbit immunoglobulin (Ig) serum (Dakopatts). Following the incubations with primary and secondary antisera, the sections were incubated with biotinylated peroxidase (biotine-HRP) together with streptavidin (both from Dakopatt's). Dilutions of antisera and washes between successive incubation steps were performed with 0.05 M phosphate buffered saline (PBS, pH 7.4–7.6) with 0.05% Tween-20 (Merck, Darmstadt, Germany).

Double immunostaining procedures consisted of a first staining sequence according to the ABC-method with a biotinylated secondary antibody (Multilink, Biogenex, San Ramon, CA, USA), streptavidin labeled alkaline phosphatase (Biogenex) and fast blue (Serva, Heidelberg, Germany) as staining reagent, and a second staining sequence according to the peroxidase-anti-peroxidase (PAP) method (60) with 3-amino-9-ethylcarbazole (AEC) (Sigma, St. Louis, MO, USA) as staining reagent. To avoid interference between the two staining sequences, the sections were incubated for 10 min in a 0.75% (w:v) M glycine-HCl buffer (0.045 M) at pH 3.0 after the first staining sequence. In the alkaline phosphatase method, endogenous phosphatase activity was blocked with 0.025% (w:v) levamisol (Sigma) and 0.01% (w:v) $NaNO_2$ (Merck).

Counterstaining of single and double immunolabeled sections was performed with Mayer's hematoxylin (Merck) for 15 s, followed by rinsing in 10% ammonia and tap water.

Cryo-immunohistochemistry

A detailed list of the mAb used for cryo-immunohistochemistry of human AP is given in Table 3. Moreover, rat mθ-specific mAbs (ED1, ED2, ED3, ED7, ED8, ED9) (34, 35) were kindly provided by Dr Christine D. Dijkstra (Free University Amsterdam). Specific antisera used for identification of rat dendritic cells and FS cells were the monoclonals OX6 and OX62 (Sera-Lab, Sussex, UK) and polyclonal anti-S100 (Dakopatts), respectively. Cryo-sections were dried for 2–3 h at room temperature and then fixed with either acetone (Merck) for 5 min, 0.012% hexazotized pararosaniline (Sigma) for 2 min (66), or with Zamboni-fixative (67) prepared as described previously (68) for 2–4 h. The latter fixation method was preferred for immuno-labeling of the diffusible cytoplasmic S100 protein (29, 32), whereas the former two fixatives were preferable for immunolabeling of membrane-bound lymphoid cell markers. In some additional experiments (4 independent preparations of 3 or 4 adult female rats) double immunolabeling was performed after fixation with freshly prepared PLP-solution (58), similar to the fixation procedure used for immuno-electron microscopy (see section below). In these experiments, the latter fixation procedure is a compromise choice for double immunolabeling of the S100 protein together with the OX6 or OX62 markers.

Single immunostaining procedures were performed according to the PAP method (cfr. above) with DAB or AEC as staining reagent. Following incubation with the primary antibodies diluted in PBS with 1% bovine serum albumin (BSA; Sigma), the cryo-sections were incubated with normal rabbit or swine sera (Dakopatts) (4% [v:v] in PBS + BSA) corresponding to the animal species in which the secondary antisera were raised, in order to prevent non-specific staining. For comparison of background staining in the different procedures, incubations with normal sera were performed preceding and subsequent to the incubation with the primary antibodies. Moreover, incubation of peroxidase(HRP)-labeled rabbit anti-mouse Ig (Dakopatts) or swine anti-rabbit Ig (Dakopatt's) was performed in the presence or absence of 4% (v:v) normal human pool serum (Bloedbank, The Hague, the Netherlands) or 4% heat-inactivated normal Wistar rat serum, for immunostaining of human or rat tissue, respectively. Human tonsillar lymphoid tissue and rat spleen or lymph nodes were used as positive control tissues. Endogenous peroxidase activity was examined in control sections treated with all incubation steps except the primary antiserum. Counterstaining with Mayer's hematoxylin and mounting of the sections was as described for the paraffin sections.

Double immunostaining procedures consisted of a combination of

TABLE 3. Immunologic Markers used for the Characterization of Cells of the Monocyte-Macrophage-Dendritic Cell Lineage Present in the Human Anterior Pituitary

CD Code	Antibodies (source)	Mol. mass	Antigen recognized	Reactivity with hematopoietic cells	References
CD1a	T6 clone 66IIc7 (Monosan, Sanbio)	gp49	T6 antigen; common thymocyte antigen LFA-1 antigen	LC, DC, B cells, cortical thymocytes lymphoid and myeloid cells	61
CD11a	LFA1/2 (CLB, Amsterdam)	gp180	LFA-1 antigen	lymphoid and myeloid cells	62
CD11b	OKM-1 (Ortho Diagnostics Systems)	gp155	MAC-1 antigen (αM-chain)	monocytes, mθ, granulocytes, NK cells	62
CD14	Leu-M3 (Becton Dickinson)	gp55	Pi-linked protein; monocytic antigen	monocytes, mθ, DC	63, 50
CD45	HLe-1 (Becton Dickinson)	gp180–220	T200 antigen; LCA	all leukocytes	50
CD68	KIM-7 (Behring)	gp110	macrophage antigen	mθ	64
–	TB1–4D5 (Ishii, Tokyo)	–	L25 antigen	DC, B cells	31
–	OKIa (Becton Dickinson)	gp 29/34	HLA-DR; MHC-class II	DC, mθ, monocytes, B cells, activated T cells	65
–	RFD-1 (Poulter, London)	–	MHC-class II associated antigen	DC, B cell (subpopulation)	44

CD Cluster of differentiation as defined during the leukocyte typing conference. gp glycoprotein. LCA Leukocyte common antigen. LFA Leukocyte function antigen. PI Phosphatidyl-inositol glycan. MHC Major histocompatibility complex. NK Natural killer cell.

the indirect HRP-labeled antibody method (cfr. above) and the alkaline phosphatase-anti-alkaline phosphatase (APAAP)-method. Staining reagents of both methods were AEC and fast blue, respectively. The cryo-sections were incubated with a mixture of two primary antibodies (listed in Table 3) and subsequently incubated with 4% normal rabbit serum (Dakopatts). Thereafter, the sections were developed in a first staining sequence by incubation with either HRP-labeled rabbit anti-mouse Ig or unlabeled rabbit anti-mouse followed by mouse APAAP (all secondary antisera from Dakopatts). Secondary antibodies were diluted in PBS with BSA and 4% (v:v) normal human pool serum (cfr. above). In a second staining sequence, the sections that had been developed according to the HRP-labeled method were further developed according to the APAAP method, and vice versa. Method specificity was tested by successively omitting the primary antiserum, the first and second staining sequence conjugates and the staining substrate.

Immuno-electron microscopy

Halved pituitaries from Wistar rats were fixed in PLP-solution (58) containing 2% (w:v) paraformaldehyde (UCB, Brussels, Belgium), 0.01 M $NaIO_4$ (Merck) and 0.075 M L-lysine (BDH Chemicals) in 0.0375 M sodium phosphate buffer. Tissue blocks were fixed for 1 h on ice, and pretreated with 2.3 M sucrose in 0.1 M PBS as a cryo-protectant (69). Cryoprotection was performed using increasing concentrations of glycerol (10%–20%–30%) (70). The tissue blocks were sandwiched with a tweezer between a small piece of plastic (Thermonox) and two low-mass copper platelets and rapidly frozen into the KF80 apparatus (Reichert). Tissue blocks were freeze substituted and embedded in the low temperature resin HM20 (Lowicryl) (70), whereafter ultrathin sections were cut for immunolabeling. Cryo-substitution of the frozen aqueous constituents of the tissue blocks was done in the CS-auto (Reichert) at −90 °C using a mixture of 0.5% uranyl acetate in methanol during 30 h. Then the temperature was raised with 4 °C per h to −45 °C and the samples were slowly impregnated in increasing concentrations of Lowicryl HM20. Polymerization took place at −45 °C using an UV light source attached to the CS-auto apparatus. After 1 to 3 days post-polymerization at room temperature, ultrathin sections were cut with the OMU III (Reichert) and transferred to copper grids for immunolabeling.

For comparing the effect of cryo-substitution and UV-polymerization upon the antigenicity of the tissue, in another series of pituitaries the Tokuyasu thawed cryo-section technique (post-sectioning immunolabeling) was used (71). Technical details of this procedure are published elsewhere (74). Not-embedded but cryo-protected tissue blocks were cut into ultrathin cryo-sections of 80–90 nm with the FC4 ultramicrotome with diamond knife at −100 °C. Immunolabeling of ultrathin cryo-sections and ultrathin resin-sections was performed according to the immunogold staining method (72).

Dilutions of antisera and immunogold complexes were made in 0.1 M PBS containing 0.05% (w:v) of the acetylated and (partly) linearized BSA combined with 0.05% (v:v) Tween-20 (Aurion BSA-C; Aurion, Wageningen, the Netherlands) in order to prevent background by hydrophobic interaction. Prior to labeling and between the incubations with primary mAbs and immunogold, the sections were rinsed 3 times in 0.2% glycine (Fluka) in 0.1 M PBS. Primary antisera used were the anti-rat MHC-class II mAb (OX6; gift of dr. N. Barclay, Oxford, UK) in dilutions between 1:2 and 1:10 and the polyclonal anti-bovine-S100 (Dakopatts), diluted 1:100 to 1:25. Immunogold staining was performed using the goat anti-mouse IgG-25 nm gold complex (Aurion) diluted 1:20. Double immunostaining was performed by first incubating the grids with the OX6 mAb followed by a rabbit-anti-mouse IgG-25 nm gold complex (Aurion) (diluted 1:20), whereafter the grids were rinsed in PBS-glycine (0.2%). For the second sequence, grids were incubated with the anti-S100 serum followed by a protein A-15 nm gold complex (Aurion) (diluted 1:20).

Contrasting and air-drying of the sections was performed according to Tokuyasu's method (73). After three rinses in distilled water the grids were placed on a drop of filtered 4% (w:v) uranyl acetate (pH 8.0) in 0.3 M oxalic acid for 3 min, and subsequently placed on a drop of 1.2% (w:v) low viscosity methyl cellulose (0.25 poise; Sigma) and filtered 1% uranyl acetate (pH 4.0) in distilled water for 3 min. Grids were examined in a Philips EM 301 electron microscope.

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