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Immunohistochemical characterization of HLA-DR-antigen positive dendritic cells in phaeochromocytomas and paragangliomas as a prognostic marker

Mutsuo Furihata and Yuji Ohtsuki

Department of Pathology, Kochi Medical School, Kochi 783, Japan

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Summary. Twelve cases of phaeochromocytoma (PCC) and four cases of paraganglioma (PGG) were studied by immunohistochemistry and immunoelectron microscopy in order to demonstrate HLA-DR (Ia)-antigen-positive dendritic cells (IaDCs). Dense infiltration of IaDCs was detected in the majority of PCCs revealing high urinary or serum catecholamine levels, but in aggressively growing PCCs, a familial PCC and all PGGs, few IaDCs were demonstrated. Interestingly, these IaDCs were negative for S-100 protein. Although S-100-protein-positive sustentacular-like cells (SCs), morphologically similar to IaDCs, were also present, these were clearly distinguished from IaDCs by our double immunostaining method. Ultrastructurally, IaDCs had smooth or slightly indented nuclei and contained a moderate amount of endoplasmic reticulum, small mitochondria and vacuoles, extending elongated cytoplasmic processes. These results suggest that determination of the quantity of IaDCs is a highly effective method of assessing the character of PCCs, in particular, their prognosis.

Key words: HLA-DR – Phaeochromocytomas – Paragangliomas – Immunohistochemistry – Ultrastructure

Introduction

In phaeochromocytoma (PCC) and paraganglioma (PGG), it is hard to predict the recurrence or the degree of malignancy from the gross and microscopic characteristics. In the adrenal PCCs, morphological criteria such as nuclear atypia, capsular and vascular invasion, and mitotic activity are generally used to predict the behaviour of tumours. It has been stated that malignant PCCs are usually larger in size, consist of rather small cells and show extensive necrotic areas (Medeiros et al. 1985). Malignant PGGs have varied considerably from case to case according to the primary site, and high mitotic activity is a consistently reliable histological criterion in assessing prognosis (Lack et al. 1980).

Although it is notoriously difficult to recognize the potentially malignant nature of the tumour on light microscopy and many cases are virtually devoid of obvious malignant features, immunohistochemical techniques have simplified the confirmation of the diagnosis and have provided important information not obtainable from a purely morphological standpoint. For example, S-100-positive sustentacular-like cells (SCs) in PCCs disappeared in the malignant cases (Schroder et al. 1986). In PGGs, the number of chief cells and S-100-positive SCs was stable despite increasing degrees of malignancy, although SCs were absent in most cases of aggressively metastasizing PGGs (Kliewer et al. 1989).

However, dendritic cell (DC) infiltration into nests of several kinds of cancers has been reported and related to their better prognosis, for example, in lung cancer (Watanabe et al. 1983; Furukawa et al. 1985; Nakajima et al. 1985; Fox et al. 1989), nasopharyngeal carcinoma (Nomori et al. 1986), epithelial skin tumours (Smolle et al. 1986), gastric carcinoma (Tsujitani et al. 1987), papillary carcinoma of the thyroid (Schröder et al. 1988), colorectal adenocarcinoma (Ambe et al. 1989) and squamous cell carcinoma of the uterine cervix (Nakano et al. 1989). Interestingly, a better survival rate was found in the patients with most DC infiltration. The DC, a group of specified cells which are dendritic in shape and have less phagocytic activity, include Langerhans' or indeterminate cells in the skin and interdigitating reticulum cells in the lymph nodes, spleen, thymic medulla or other organs (Wood et al. 1985;, Franklin et al. 1986). DCs are also characterized immunohistochemically by the expression of HLA-DR antigen (Rowden et al. 1977; Klareskog et al. 1977; Steinman et al. 1979). The S-100 protein is also a distinguishing marker of some DCs from conventional histiocytes of monocyte-macrophage lineage (Takahashi et al. 1981; Nakajima et al. 1982). In particular, Langerhans' cells con-

Offprint requests to: M. Furihata

tained characteristic Birbeck granules, expressed the antigen defined by OKT6 (Fithian et al. 1981) and bore Fc and C3 receptors (Stingl et al. 1977). These investigations suggested that DC infiltration in various kinds of cancer have an important immunological significance in the host-defence mechanism.

This paper deals with the detailed characterization of HLA-DR-positive and S-100-negative dendritic cells infiltrating densely in PCCs, employing immunohistochemical and ultrastructural techniques. In addition, we briefly discussed the IaDC infiltration into each PCC in relation to their progression and prognosis.

Materials and methods

We investigated 12 cases of PCCs, composed of nine benign PCCs, a case of familial PCC and two cases of aggressively growing PCCs. We also studied four PGGs: one each from the carotid body, back, retroperitoneum and thorax. These patients, ranging from 26 to 80 years old, comprised six men and ten women (Table 1). The tumours used were surgically removed or were obtained at autopsy.

Formalin-fixed paraffin-embedded tissues were studied using following antibodies: monoclonal antibodies to HLA-DR alpha chain (IaA, dilution 1:30); T cell CD45R (UCHL-1, 1:25); B cell L26 (L26, 1:25); leucocyte common antigen (LC, 1:30); vimentin (Vim, 1:50) and polyclonal antibodies to S-100 protein (S-100, 1:800); lysozyme (LYZ, 1:400); alpha-1-antichymotrypsin (ACT, 1:400); factor VIII (Fac VIII 1:400); glial fibrillary acidic protein (GFAP 1:200); all of these antibodies had been purchased from Dakopatts (Copenhagen, Denmark).

Deparaffinized sections were washed in phosphate buffered saline (PBS; pH 7.4) successively incubated with 10% normal goat serum (NGS), incubated at 4° C overnight with appropriately diluted primary antisera. After washing in PBS, the sections were exposed to the goat anti-mouse or anti-rabbit biotinylated secondary antibody for 30 min, washed with PBS, and treated with an avidin-biotin complex alkaline phosphatase (ABC-AP) kit (Vector Laboratory, CA, USA) as described in the kit manual. After washing, the enzyme reaction was developed with alkaline phosphatase substrate system kit I (Vector Laboratory, CA, USA) for 20 min. After alkaline phosphatase reaction, the sections were washed with distilled water and counterstained with methyl green (Wako Pure Chemicals, Osaka, Japan).

For double immunostaining for S-100 and IaA, deparaffinized sections were treated with 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. After washing with PBS, the sections were successively incubated with 10% NGS and diluted antisera to S-100 protein washed with PBS, and further treated with HRPO-conjugated goat Fab fragment to rabbit IgG (1:60) (MBL, Tokyo, Japan). Each incubation was performed for 60 min at room temperature. After washing, the peroxidase reaction was developed with 3-3′-diaminobenzidine 4 HCl solution with 0.01% H_2O_2 for 5 min. After peroxidase reaction, the sections were washed with distilled water. After washing with PBS, on immunoalkaline phosphatase method using an ABC-AP was performed for the immunoreactivity of the IaA as described above.

We examined the maximum cut-surface of each tumour. The number of IaDCs or SCs was counted with a ×40 objective, in the five selected high power fields in which these cells infiltrated most densely a mean was determined (BHT Olympus, Tokyo, Japan). The cases were divided into two groups: "few" means less than 20 cells and "many" means more than 20 cells.

Control sections stained in parallel included replacement of the specific antibody by PBS, and staining of known positive or negative tissues for each antibody used.

For electron microscopy, small fragments of the PCC fixed with buffered formalin; which had been demonstrated to have dense IaDC infiltration immunohistochemically, were fixed in PLP (periodate-lysine-paraformaldehyde) solution for 10 h at 4° C and treated with first 10%, then 15% and finally 20% sucrose solution in PBS. These tissue blocks were quickly frozen, semithin sections cut and processed for the immune reaction by the ABC peroxidase method, using IaA antibody as a primary reagent. The sections were fixed with 2% glutaraldehyde for 30 min and post-fixed in 2% OsO_4 for 45 min at 4° C, dehydrated through a series of graded ethanol, and embedded in an epoxy resin. Ultrathin sections were cut and examined under an H-300 type Hitachi electron microscope. These ultrathin sections were observed without counterstaining.

Table 1. Clinical details of the tumours

Case no.	Age (years)	Sex	Tumour	Site	Weight	Follow-up study
1	47	M	PCC(Ben)	L.Ad	31 g	10 years, alive
2	50	F	PCC(Ben)	L.Ad	20 g	3 years, alive
3	41	F	PCC(Ben)	R.Ad	56 g	4 years, alive
4	69	M	PCC(Ben)	L.Ad	760 g	2 years, alive
5	41	F	PCC(Ben)	L.Ad	40 g	3 years, alive
6	38	F	PCC(Ben)	R.Ad	100 g	5 years, alive
7	32	F	PCC(Ben)	R.Ad	41 g	8 years, alive
8	29	M	PCC(Ben)	R.Ad	35 g	9 years, alive
9	43	M	PCC(Ben)	L.Ad	111 g	4 years, alive
10	28	F	PCC(Fam)	R.Ad	60 g	4 years, alive
11	26	F	PCC(Ag)	R.Ad	nd	1 year, died
12	38	M	PCC(Ag)	Ret	nd	Autopsy material
13	27	F	PGG(Ben)	R.Car	nd	4 years, alive
14	46	F	PGG(Mal)	R.Bac	nd	2 years, alive
15	80	F	PGG(Mal)	R.Ret	1140 g	Autopsy material
16	60	M	PGG(Mal)	R.Tho	nd	1 year, died

PCC, Phaeochromocytoma; PGG, paraganglioma; Ad, adrenal gland; Car, carotid body; Bac, back; Ret, retroperitoneum; Tho, thorax; Ben, benign; Mal, malignant; Fam, familial; Ag, aggressively growing

Results

The IaDC or SC infiltration in each PCC or PGG is summarized in Table 2. Dense infiltrates of IaDC were seen in the nine benign PCCs (cases 1–9), while in a familial one (case 10), in the two cases of aggressively growing tumours (cases 11, 12) and the remaining PGGs (cases 13–16), little infiltration was noted. The cells, scattered throughout the tumour tissue, were identified by positive immunoreactivity with IaA, revealing their dendritic morphology (Fig. 1a, b). They were observed mainly in the intercellular space of the tumour cells and were negative for Vim, Fac VIII and GFAP. LC-positive lymphocytes and LYZ- or ACT-positive macrophages were also observed. The majority of lymphocytes were T cells stained positively with UCHL-1, intermingling few L-26-positive B cells. SCs, distinctly stained with S-100, were recognized in their morphology to be similar to IaDCs; however, they extended thinner and more elongated processes than those of IaDCs (Fig. 1c). Double immunostaining techniques were then performed to identify both IaDCs and SCs, resulting in distinct positivity of IaDCs for HLA-DR alpha chain (red) and SCs for S-100 protein (brown) (Fig. 1d). Generally, in the parts of PCCs predominantly consisting of spindle-shaped cells associated with ganglioneuromatous foci, scanty infiltration of IaDC was observed. SCs were present in all cases of PGG and PCC, except for the two cases (nos. 11, 12) of aggressively growing PCCs.

In immunoelectron microscopic study of IaDCs reacted with IaA in a case of PCC, immunoreactivity of the positively stained dendritic cells was detected not only in the plasma membrane but also in the cytoplasmic matrix (Fig. 2). The cells had round or oval heterochromatin-rich nuclei containing small nucleoli, and extended several elongated cytoplasmic dendritic processes. The cytoplasm of the labelled cells was characterized by a moderate amount of rough endoplasmic reticulum and occasional small mitochondria, both of which showed no immunoreactivity. The nuclear membrane was also negative, as shown in Fig. 2a and b. These cells were devoid of basal laminae and attached directly to the tumour cell surface associated with the collagen fibres on the other side.

Control samples of PCCs or PGGs treated with non-immune sera exhibited no reaction product, as shown by both light and electron microscopic observation.

The laboratory data for serum or urinary catecholamines and vanillylmandelic acid (VMA) levels are summarized in Table 3. In contrast with the normal adrenal medulla, most PCCs secrete noradrenaline predominantly rather than adrenaline. All the PCCs except for case 7 revealed a more elevated serum or urinary noradrenaline and/or dopamine level than for adrenaline. In particular, three cases (nos. 5, 9 and 10) showed high levels of dopamine and noradrenaline, but very low levels of adrenaline. The episodes of paroxysmal or sustained hypertension, and hypermetabolism (an elevated basal metabolic rate, hyperglycaemia, and elevated plasma free

Table 2. The distribution and density of IaDCs and SCs

	IaDCs		SCs	
	Many[a]	Few[b]	Many[a]	Few[b]
No. of cases	9	7 4PGGs	14 4PGGs	2
(Case no.)	(1–9)	(10–16)	(1–10, 13–16)	(11, 12)

IaDC, HLA-DR-positive dendritic cell; SC, sustentacular-like cell; PCC, phaeochromocytoma; PGG, paraganglioma
[a] Mean no. of IaDCs or SCs/5HPF (>20, =20 cells)
[b] Mean no. of IaDCs or SCs/5HPF (<20 cells)

Table 3. Laboratory data on the tumours

Case no.	s.Ad pg/ml	u.Ad μg/24 h	s.Nd pg/ml	u.Nd μg/24 h	s.Dp pg/ml	u.Dp μg/24 h	VMA μg/24 h
1	ND	141	ND	345	ND	ND	ND
2	ND	105	ND	655	ND	1098	ND
3	2500[a]	ND	21200[a]	ND	<200[a]	ND	ND
4	ND	239	ND	7840	ND	3860	130
5	49	9	6660	437	<200	3940	3
6	16	427	293	1140	<300	620	9
7	619	274	672	195	<300	320	15
8	40	102	4130	1700	<200	2650	37
9	ND	17	ND	221	ND	3020	21
10	3	10	252	80	<200	1450	9
11	ND	ND	ND	ND	ND	ND	ND
12	ND	ND	ND	ND	ND	ND	ND
13	14	ND	68	ND	660	ND	5
14	ND	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	ND	ND
16	ND	ND	ND	ND	ND	ND	ND

s.Ad, Serum adrenaline; u.Ad, urinary adrenaline; s.Nd, serum noradrenaline; u.Nd, urinary noradrenaline; s.Dp, serum dopamine; u.Dp, urinary dopamine; VMA, vanillylmandelic acid
[a] Directly sampled from the superior vena cava

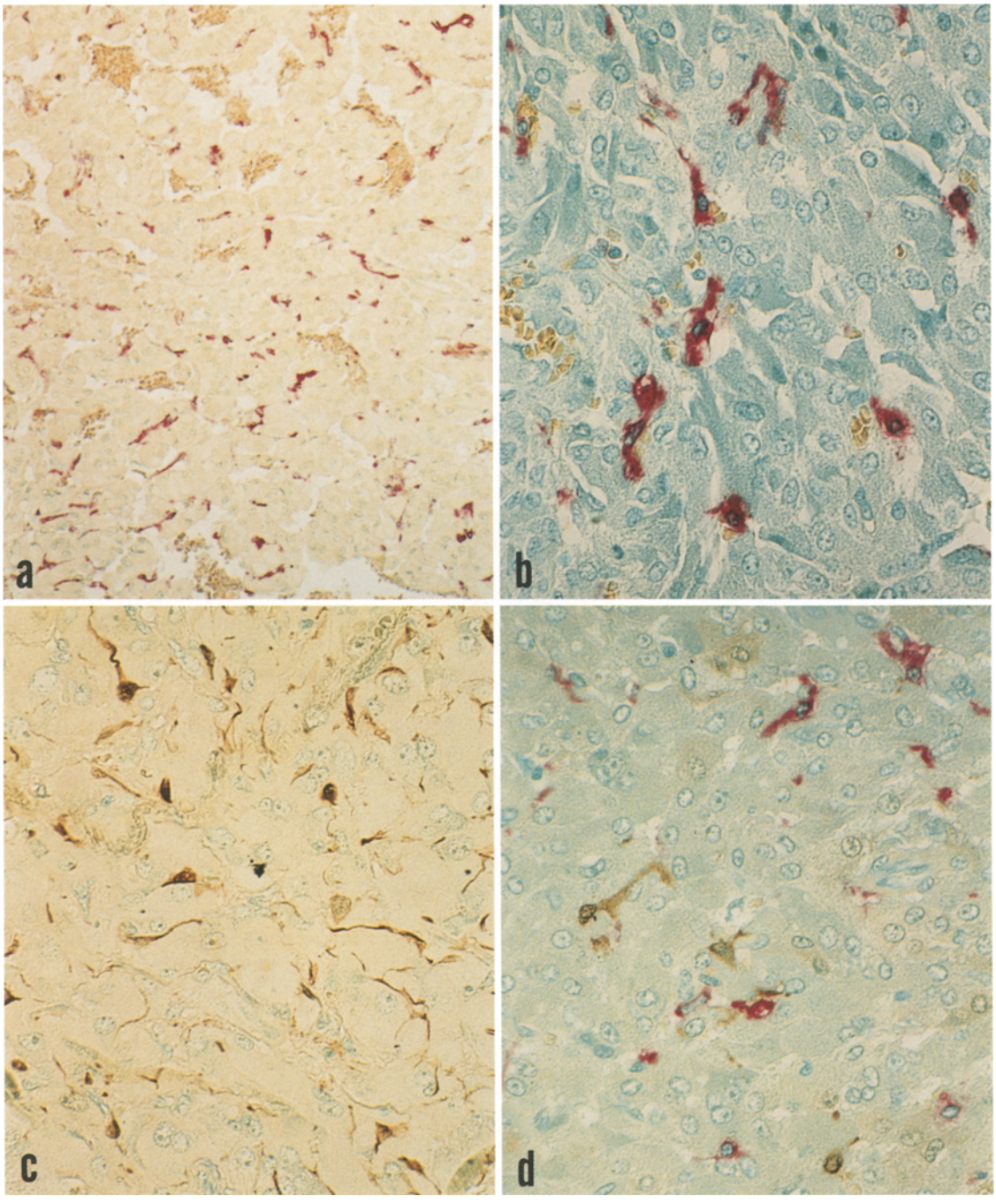

Fig. 1. a A general view of HLA-DR (Ia)-antigen-positive dendritic cell (IaDC) infiltration among the tumour cells in a case of phaechromocytoma (PCC). ABC immunostain, ×160. **b** IaDCs morphologically similar to Langerhans' cell. ABC immunostain, ×400. **c** Spindle-shaped or stellate S-100 positive sustentacular-like cells (SCs) scattered diffusely in a case of PCC. Indirect immunostain, ×400. **d** The immunoreactivity of IaDCs (*red*) with antibody to HLA-DR alpha chain and of SCs (*brown*) with antibody to S-100. Note the precise staining of each cell type. Double immunostain, ×400

fatty acids) had also been noticed in nine benign cases. However, neither these clinical signs nor symptoms were recognized in the remaining PCCs and PGGs.

As shown in Table 1, nine patients with benign PCCs are all alive in 2–10 years follow-up study, while two patients with aggressive PCCs have died. Malignant PGGs also showed poor prognosis. Case 14 is now in a poor clinical condition, although alive.

Discussion

The present study has shown that the IaDC can be readily identified immunohistochemically in benign PCCs, even though they are absent or extremely rare in normal adrenal medulla and paraganglia (Natali et al. 1981). In double immunostaining with IaA and S-100, it was demonstrated that IaDCs were negative for S-100, as shown

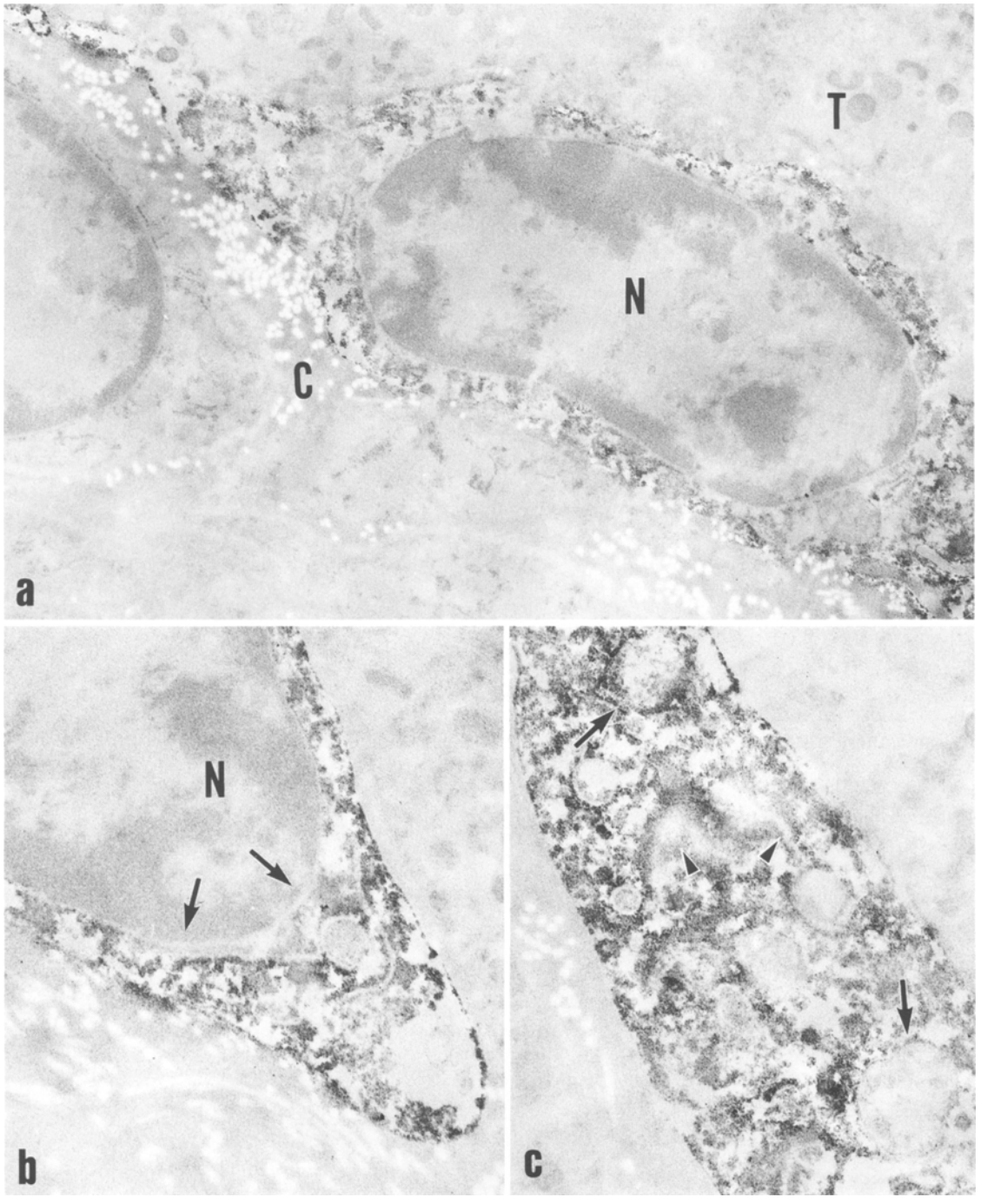

Fig. 2. **a** An immunoelectron micrograph of IaDC reacted with antibody to HLA-DR alpha chain. The positive cell possesses an oval nucleus (*N*), showing the direct contact with the tumour cell (*T*) associated with the stromal collagen (*C*) on the other side. The positive immune reaction is recognized in the plasma membrane and cytoplasmic matrix. ×19,000. **b** The nucleus (*N*) and nuclear envelope (*arrows*) are not reacting. ×38,000. **c** Mitochondria (*arrows*) and rough endoplasmic reticulum (*arrowheads*) are also negative. ×38,000

in Fig. 1d. Therefore, the difference between IaDCs and SCs was obvious. To distinguish IaDCs from macrophages, some inflammatory cells, endothelial cells and fibroblasts, we used several antibodies in immunohistochemical staining and revealed that the distribution and/or morphology of IaDCs differed from those cells. In immunoelectron microscopy of buffered formalin-fixed material, we were unable to examine the cells precisely to find Birbeck granules, pinocytotic vesicles or cell attachment. Therefore, it is difficult to determine the characteristics of IaDCs based on these data, although their expression of HLA-DR antigen and dendritic form in morphology are phenotypic features characterizing the dendritic cell family. Dendritic cells generally exhibit several characteristic features which distinguish them from histiocytes of monocyte-macrophage lineage. These in-

clude dendritic morphology, more potent accessory cell function, minimal phagocytic activity, and lower levels of lysosomal enzymes (Van Voorhis et al. 1983; Dimitrin-Bona et al. 1983). Ultrastructurally, dendritic cells contain relatively few cytoplasmic organelles related with phagocytic function, while conventional histiocytes of monocyte-macrophage lineage are rich in lysosomes and cytoplasmic vacuoles (Van Voorhis et al. 1982).

Although the functions of dendritic cells remain controversial, the fact that they express HLA-DR antigen and that Langerhans' cells bear Fc and C3 receptors suggests that they act as antigen presenting cells (APCs) and stimulate antigen-specific T-lymphocyte (Poulter et al. 1983). So-called APCs are generally thought to present antigens to T-cells (Inaba et al. 1984, 1985; Kapsenberg et al. 1986) and are thus involved in T-cell activation in conjunction with the presence of HLA-DR antigen and of interleukin 1. As T-cells play a major role in antitumour cytotoxicity, IaDCs infiltrating the tumour are assumed to represent an ongoing immune response of the host it might be predicted that dense infiltration of IaDCs would be protective for tumour growth or at least react as a prognostic factor to improve the survival rate.

We have reported here the detailed distribution of S-100-protein-negative IaDCs in PCCs, which has not previously been reported. The current study also indicates that the quantity of IaDC infiltration in tumours is a highly effective means of assessing the prognosis of patients with PCCs. In fact, in our PCCs showing the dense infiltration of IaDCs all patients are now well on follow-up with no signs of recurrence or metastasis. However, two patients with aggressively growing PCCs died. In general, most cases of PCCs follow a benign clinical courses and the quoted incidence of malignancy is within about 10%. However, long-term follow-up studies have suggested that the real incidence of malignancy may be higher. In addition, there are no reliable morphological criteria for malignancy. Therefore, the demonstration of IaDCs may be of value in predicting the behaviour of PCCs.

From an analysis of a correlation between the density of IaDCs and several clinical factors, prominent IaDC infiltration was detected in the cases of PCC showing abnormally high serum or urinary catecholamines and VMA levels (Table 3), but not in the other cases. Elevated serum catecholamine level in PCCs may be likely to have an intimate relationship with IaDC infiltration.

IaDC infiltration into PCCs may play an important immunological role in host defense mechanisms, and may act in this way as one of the prognostic factors.

Our limited study allows us to say only that the dense infiltration of HLA-DR-positive, S-100-negative dendritic cells in benign PCCs is not seen in the aggressive PCCs and PGGs tested. Further studies to characterize the IaDC and estimate more precise quantitative relationship between the degree of IaDC infiltration and the prognosis are also required.

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