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DISTRIBUTION OF C-CELLS IN PARATHYROID GLAND IV AND THYMUS IV OF DIFFERENT MAMMALS STUDIED BY IMMUNOPEROXIDASE METHOD USING ANTI-CALCITONIN AND ANTI-C-THYROGLOBULIN ANTISERA

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Abstract

In order to determine whether or not the C-cells are distributed in parathyroid glands and thymuses as well as in thyroid glands, the thyroid glands with surrounding tissues of ten rabbits, sixteen cats, three goats and ninety-two dogs were cut into the complete serial sections, and examined by immunoperoxidase method using the specific antisera to human calcitonin, porcine calcitonin and canine C-thyroglobulin. The C-cells of each animal species were filled with numerous immunoreactive secretory granules after the specific immunoperoxidase staining and distinguished easily from nonreactive thyroid follicular cells, parathyroid cells and thymic cells. In rabbits, cats and goats the C-cells were almost constantly observed in the parathyroid glands IV. A large number of C-cells were widely dispersed among the parathyroid cells as single cells or as small clusters of cells. Of 92 dogs examined, nine revealed the presence of C-cells in parathyroid IV. Their distribution was mainly restricted to the peripheral regions of parathyroid parenchyma. Thymus IV was frequently observed adjacent to parathyroid IV in thyroid lobes of cats and dogs. Ten out of 12 thymuses IV in cats and fourteen out of 15 thymuses IV in dogs revealed the distribution of C-cells. No C-cells were found in the parathyroid gland III and thymus III. In conclusion, the C-cells of some mammals are distributed not only in the thyroid gland but also in the parathyroid gland IV and thymus IV intimately associated with the ultimobranchial bodies during the fetal period. That is, the origin of calcitonin is not restricted to the thyroid gland, though it is usually the most significant source.

亀田芙子

INTRODUCTION

The thyroid parafollicular (C) cells synthesize and secrete calcitonin, a serum calcium-lowering polypeptide hormone. The C-cells are not uniformly or randomly distributed throughout the thyroid glands. In many mammalian species, they reveal the tendency to be concentrated around parathyroids IV (internal parathyroid glands)[1,2]. Furthermore, it has been reported that in some species, i.e., the rabbit, dog and cat, the C-cells are present in parathyroid IV and thymus IV[1—5]. These studies have used, however, histological and histochemical methods for the demonstration of C-cells such as silver impregnation, basic dyes and lead-hematoxylin stainings or cholinesterase reactions. On the other hand, the recent immunoperoxidase stainings using anti-calcitonin antiserum which are specific for calcitonin in contrast to nonspecific and less sensitive histochemical techniques have given conflicting data that C-cells are not distributed in the parathyroid gland and thymus[6—9]. In order to test whether or not C-cells are also distributed in parathyroids and thymuses, the present study applies the immunoperoxidase method using the specific antisera to human calcitonin, porcine calcitonin and dog C-thyroglobulin (C-Tg) to the systematic investigations of distribution of C-cells in thyroids, parathyroids and thymuses of rabbits, cats, goats and dogs. Furthermore, the immunoreactivity of each antiserum to C-cells of these animals is investigated.

C-Tg is the largest molecular weight component (MW approx. 2,600,000) of thyroglobulin which is composed of several components[10]. Antiserum to C-Tg has two sorts of antigenicity ; it reacts to follicular cells and follicular colloid as does antiserum to 19S-thyroglobulin, and also reacts to secretory granules of C-cells as does antiserum to calcitonin. The relationship between calcitonin and C-Tg has been described in the previous studies : 1) C-Tg and calcitonin antisera cross-react to a certain degree[10] ; 2) the reaction of anti-C-Tg antiserum in fetal C-cells appears at earlier stages and more strongly than that of anti-calcitonin antiserum[11] ; 3) tumour cells in medullary thyroid carcinoma, a distinct neoplasm derived from C-cells, reveal a far stronger immunoreaction for C-Tg than for calcitonin[12]. These data strongly suggest that C-Tg molecule contains the specific peptide chain composition corresponding to the biosynthetic precursor of calcitonin.

MATERIALS AND METHODS

Ten rabbits, sixteen cats, three goats and ninety-two dogs of either sex and various ages were used. The dogs under several experimental conditions used in the previous studies[13—15] were included in these. Thyroid glands were fixed in Bouin's solution and GPA solution (25% glutaraldehyde, 1 vol., saturated

aqueous solution of picric acid, 3 vol., and acetic acid to give 1%) for 24–48 hrs. The specimens were embedded in paraffin. One thyroid lobe from each animal was cut into longitudinal total serial sections 7–10 μm in thickness. The other lobe was cut in 5μm nonserial sections. Besides hematoxylin-eosin and PAS stainings for general purposes, the silver impregnation[16], pseudoisocyanin[17] and lead-hematoxylin[18] stainings were employed for the histological demonstration of C-cells. For immunological staining, an unlabeled antibody-enzyme bridge technique was used as previously described[19]. The following primary antisera were employed : anti-synthetic human calcitonin, anti-extracted porcine calcitonin, anti-dog C-Tg and anti-dog 19S-thyroglobulin antisera. Synthetic human calcitonin (Peptide Institute Protein Research Foundation, Osaka) was kindly made available by Yamanouchi Pharmaceutical Co. The antibody to synthetic human calcitonin was produced as follows ; the hormone (total amount 0.54 mg per animal), not conjugated with a carrier protein, was emulsified with Freund's complete adjuvant and repeatedly injected subcutaneously into the back of the neck of rabbits. The preparation and serological studies of other antisera have been described previously[10,20]. The sections were reacted with the following sequence of solutions after hydration with phosphate-buffered saline (PBS) : an appropriate dilution of the primary antisera (1 : 10 to 1 : 2,000) for 5 hrs, goat anti-rabbit globulin antiserum (1 : 10) for 15 min, and rabbit anti-peroxidase antiserum (1 : 10) for 15 min. After incubation in horseradish peroxidase solution (0.5 mg/100 ml) containing 0.1% bovine serum albumin, reaction products were developed with 3, 3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) and H_2O_2 (0.01%). Sections were washed three times for 7 min each with PBS between the steps. All reactions were carried out at room temperature. Specificity of immunostaining was assessed by two procedures ; 1) the primary antisera were replaced with a nonimmune serum, and 2) the primary antisera were absorbed before use with various amounts of the respective antigens (human calcitonin, porcine calcitonin, dog C-Tg and dog 19S-thyroglobulin).

RESULTS

C-cells of thyroid gland

In all animal species used, rabbits cats, goats and dogs, the C-cells were specifically stained by immunoperoxidase method using the antisera against human calcitonin, porcine calcitonin and dog C-Tg, respectively. The C-cells were evident due to the numerous immunoreactive secretory granules filling the cytoplasm of the cell. The specificity of staining was verified by its abolition after absorption of the anti-calcitonin and anti-C-Tg antisera with respective

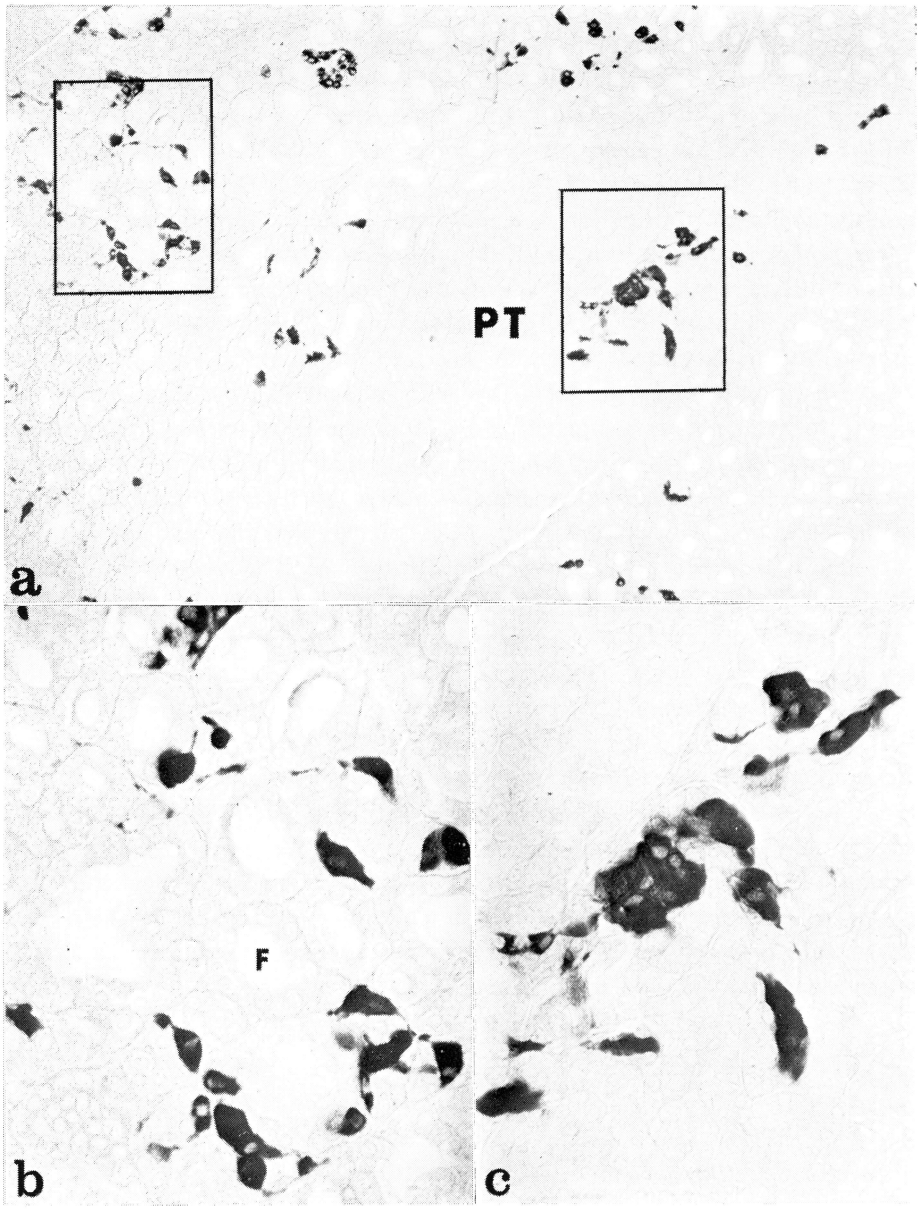


Fig. *1a*. Parathyroid gland IV (internal parathyroid) included in thyroid parenchyma of a rabbit, stained by immunoperoxidase method using anti-human calcitonin antiserum. The C-cells alone are specifically stained. The immunoreactive C-cells are distributed not only in thyroid but also in parathyroid (PT). ×90

1b. Higher magnification of the insert of thyroid parenchyma in 1a. The C-cells are filled with numerous immunoreactive secretory granules. They are oval or elongate in shape and located in para- and intrafollicular positions. F, follicle. ×290 *1c.* Higher magnification of the insert of parathyroid in 1a. The clusters of C-cells, showing the same morphological characteristics and immunoreactivity as thyroid C-cells, are dispersed among parathyroid cells. ×290

antigens and by substitution of normal rabbit serum in the place of the primary antisera. Furthermore, when antiserum to 19S-thyroglobulin was used, no staining of C-cells was observed.

The staining intensity of each antiserum was species variable. Anti-human calcitonin antiserum reacted strongly to rabbit C-cells but faintly to the cells of other species, whereas anti-porcine calcitonin and anti-dog C-Tg antisera reacted strongly to the C-cells of cats, goats and dogs. Although Bouin's solution was the best fixative, the specimens fixed in GPA solution could be also used for the immunoperoxidase staining of C-cells. They, however, revealed higher background reactions and required more increasing concentrations of the antisera than those fixed in Bouin's solution. The specimens used in the previous studies[1,2], fixed in GPA solution and stained by histological staining methods, i.e., silver impregnation, pseudoisocyanin and lead-hematoxylin, were also available to compare the immunoperoxidase stainings and histological stainings of the C-cells. The morphological characteristics of C-cells of each animal identified by the immunoperoxidase staining as well as their localization and distribution were completely identical with those demonstrated by the histological stainings.

The shape, size and distribution pattern of C-cells in thyroid glands varied from species to species. In rabbits, C-cells appeared as single cells or as a small group of cells localized close to the basal portion of the follicular epithelium (Fig. 1a, b). The cells were oval or elongate in shape and often possessed delicate cytoplasmic protrusions (Fig. 1b). The distribution of C-cells was restricted mainly around parathyroid gland IV. This zone was consistently found in the middle one third of the thyroid lobe and outside this zone of concentration the frequency of occurrence of C-cells diminished rapidly. Consequently, the superior and inferior poles were devoid of C-cells. In cats, C-cells were oval or round in shape and always grouped in small cell clusters (Fig. 2a, b, c). They were located in para- and interfollicular positions. The C-cells were mainly distributed in the upper two thirds of the thyroid, as parathyroid IV of cats was usually localized in the upper part of thyroid lobe. In goats, C-cells were conspicuously elongate in shape and possessed very long

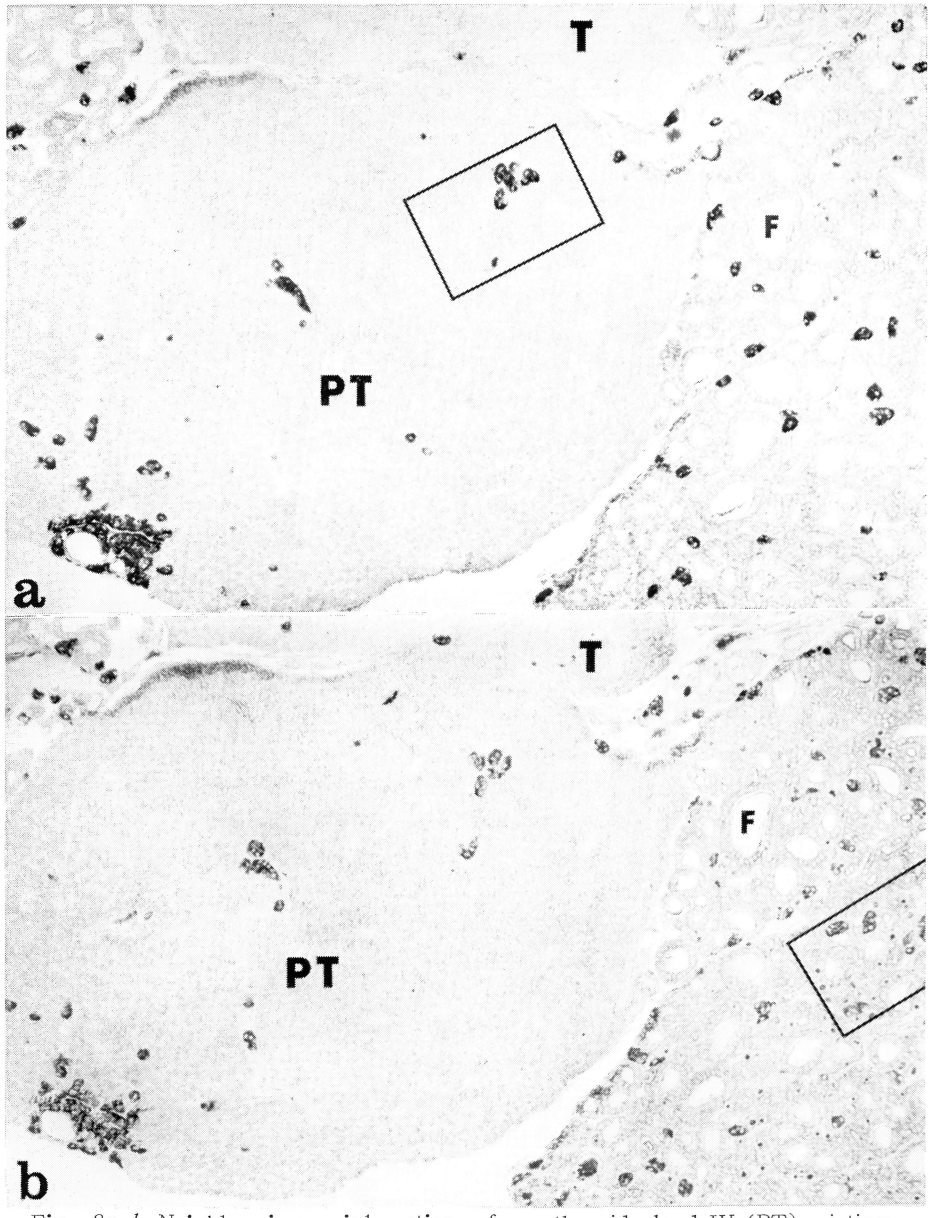
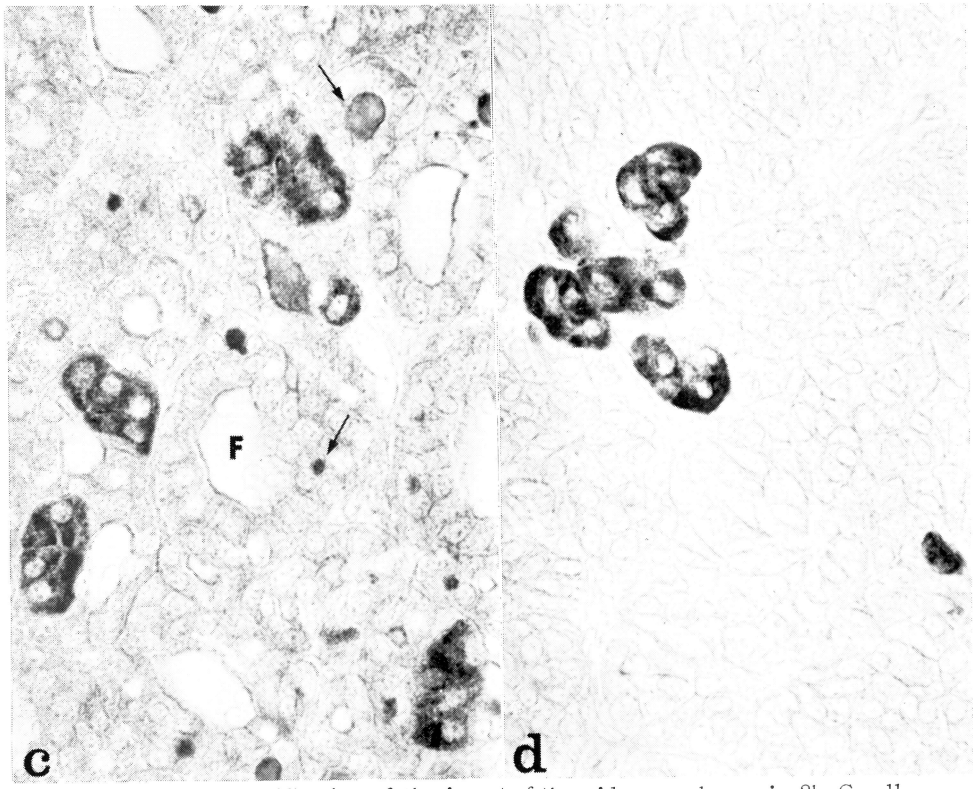


Fig. *2a, b.* Neighbouring serial sections of parathyroid gland IV (PT) existing in thyroid gland of a cat, stained by immunoperoxidase method using two kinds of antisera. Thymus IV (T) is present in the upper part of the pictures. F, follicles in thyroid. ×90 *a.* Reaction of anti-porcine calcitonin antiserum. C-cells, packed with stained substances and gathered in small cell clusters, are also distributed in the parathyroid. *b.* Reaction of anti-dog C-Tg antiserum. The antiserum shares the common immunoperoxidase reaction to C-cells with anti-calcitonin antiserum.



2c. Higher magnification of the insert of thyroid parenchyma in 2b. C-cells are filled with numerous reaction products for C-Tg. Although the antiserum was absorbed with 19S-thyroglobulin, the immunoreaction of colloid in primordial follicles (arrows) still remains strongly. F, follicle. ×460 *2b*. Higher magnification of the insert of parathyroid IV in 2a. The small clusters of C-cells are dispersed among parathyroid cells. ×460

and delicate cytoplasmic protrusions (Fig. 3a). In sections where the cells were cut transversely, they appeared as very small cells with round to oval cytoplasm. The C-cells occupied the intra- and parafollicular positions, with the latter predominating, and were usually scattered in the form of single elements. The distribution of C-cells was limited to the upper two thirds of the thyroid lobe. In dogs, the C-cells tended to be concentrated in the dorsomedial portion of thyroid gland, surrounding the region of the parathyroid IV, although the cells were abundantly distributed throughout the whole thyroid gland. The cells were oval or round in shape and grouped mostly in cell clusters. They had inter- and parafollicular locations, and further intrafollicular location. The characteristic histological and immunocytochemical features of canine C-cells have

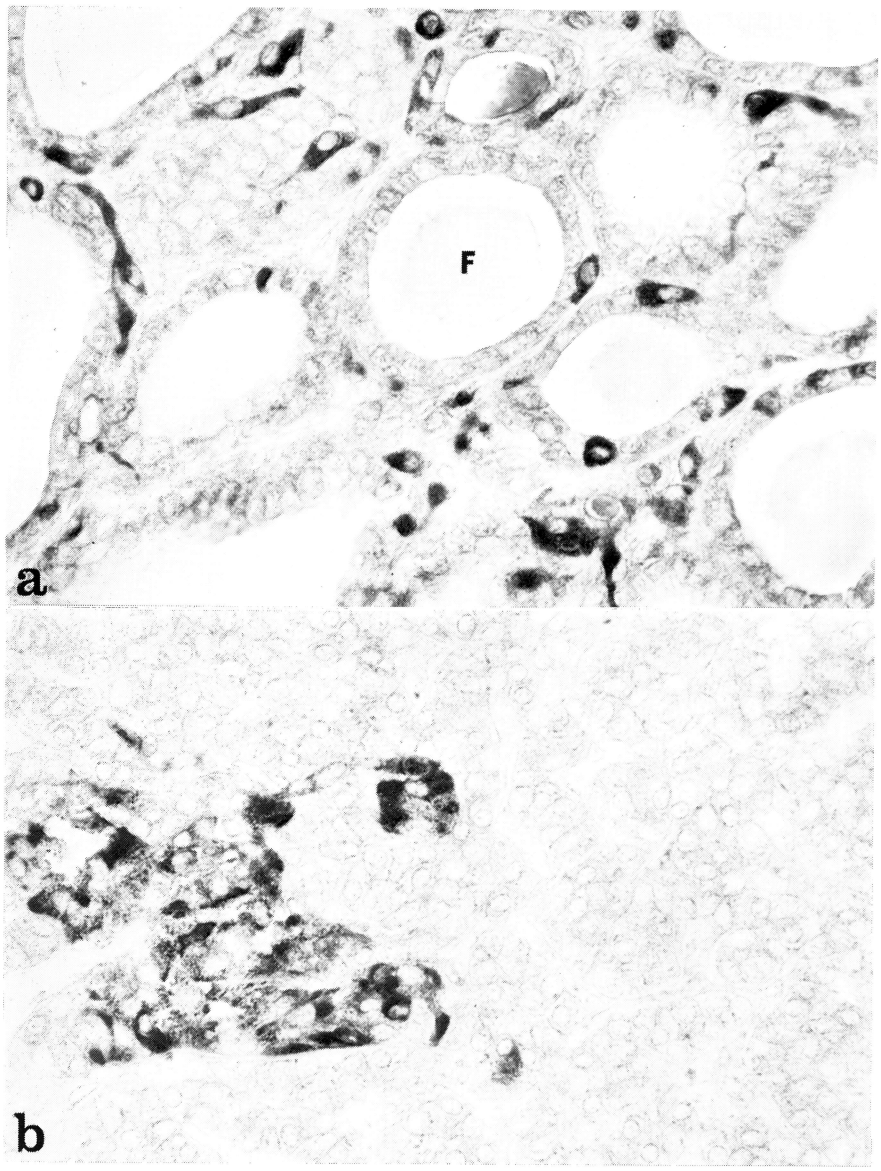


Fig. *3a*. Thyroid gland of a goat stained by immunoperoxidase method using anti-C-Tg antiserum. C-cells possessed long cytoplasmic protrusions are scattered as single cells in para- and intrafollicular positions. Although the antiserum was absorbed with 19S-thyroglobulin, the faint reactions of follicular cells and follicular colloid are still observed. F, follicle. ×360
3b. Parathyroid gland IV of a goat stained by immunoperoxidase method using anti-C-Tg antiserum. The C-cells with elongate cytoplasm are gathered in large cell clusters among parathyroid cells. ×360

been well investigated by the author[1,5,10,19,21].

Distribution of C-cells in parathyroid gland IV

When the immunoperoxidase staining using anti-human calcitonin, anti-porcine calcitonin and anti-dog C-Tg antisera was applied to tissue sections of parathyroid gland IV of each animal species, the C-cells were also found in them. The C-cells in parathyroid IV as well as in thyroids were filled with numerous reaction products after the immunoperoxidase staining and distinguished easily from nonreactive parathyroid cells. In all animal species, rabbits, cats, goats and dogs, the C-cells distributing in parathyroid IV revealed the same sensitivity to each antiserum as the cells in thyroids. In rabbits the C-cells in parathyroid IV were densely stained with anti-human calcitonin antiserum (Fig. la, c), whereas in cats, goats and dogs the cells were stained densely with anti-porcine calcitonin and anti-dog C-Tg antisera and weakly with anti-human calcitonin antiserum (Fig. 2a, b, d, 3b and 4). The C-cells in parathyroid IV stained by the immunoperoxidase method were completely identical in their features and distribution patterns with those stained by histological staining methods such as silver impregnation, pseudoisocyanin and lead-hematoxylin.

In rabbits, cats and goats the C-cells were almost constantly observed in parathyroid IV. The cells were distributed abundantly and diffusely throughout the whole parathyroid parenchyma. When ten specimens of parathyroid IV included in thyroid lobes of rabbits were examined, nine revealed the presence of C-cells (Fig. 1a). In sixteen cats and three goats, all cases revealed the distribution of C-cells in parathyroid IV (Fig. 2a, b and 3b). In contrast to these animal species, dogs represented a low frequency of occurrence of C-cells in parathyroid IV ; nine only out of 92 dogs contained C-cells in parathyroid IV. The frequency of occurrence was 9.8%. Furthermore, the cells were mainly localized in the peripheral regions of parathyroid parenchyma and a small number (Fig. 4).

The C-cells in parathyroid IV shared the common shape, size and distribution pattern with those in thyroids, and they differed from species to species. That is, in rabbits C-cells were distributed as single cells or as small clusters among the parathyroid cells (Fig. 1c). They were oval or elongate in shape and often possessed cytoplasmic protrusions. In cats the cells with round to oval cytoplasm were always gathered in cell groups and mingled with the parathyroid cells (Fig. 2d). In goats the C-cells were scattered within parathyroid parenchyma as solitary cells, though they formed occasional large aggregations (Fig. 3b). The cells were elongate in shape. The C-cells of dogs

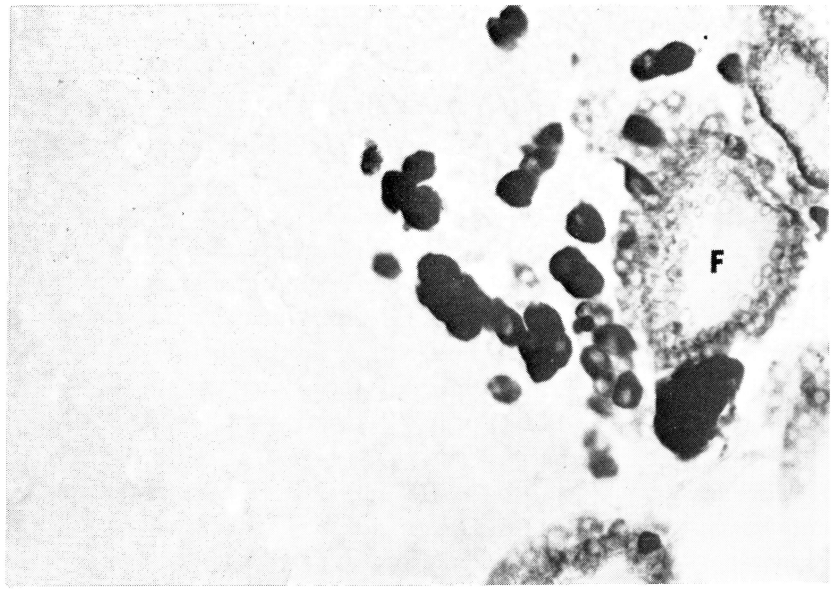


Fig. 4. Parathyroid gland IV of a dog stained by immunoperoxidase method using anti-C-Tg antiserum. The periphery of the parathyroid is fused with thyroid parenchyma and reveals the distribution of C-cells. Thyroid follicles (F) displaying dense immunoreaction. ×290

showing plenty cytoplasm and a large nucleus were distributed as cell clusters or rarely as single cells in the parathyroid (Fig. 4).

Distribution of C-cells in thymus IV

In thyroid glands of cats and dogs, thymus tissues were often observed adjacent to the parathyroid IV. They are regarded as thymus IV, because the thymus IV of the cat and dog is derived from the pharyngeal pouch IV together with parathyroid IV and ultimobranchial body[22,23].

In the complete serial sections of 16 thyroid lobes of cats, twelve cases contained thymus IV. Ten out of the 12 thymuses IV revealed the distribution of C-cells (Fig. 5a). In 92 thyroid lobes of dogs, there were fifteen with thymus tissues in connection with parathyroid IV. Except one case in which thymus tissue was very small, the C-cells were constantly observed in the thymus IV (Fig. 5b). The C-cells in thymus IV of these animals were characterized with the same cytological features and immunoreactions to each antiserum as the cells in thyroids and parathyroids IV. The cysts of various sizes which contained colloid-like substances and were covered with stratified epithelium were often observed in the medullary portion of the thymus. C-cells were mainly distributed

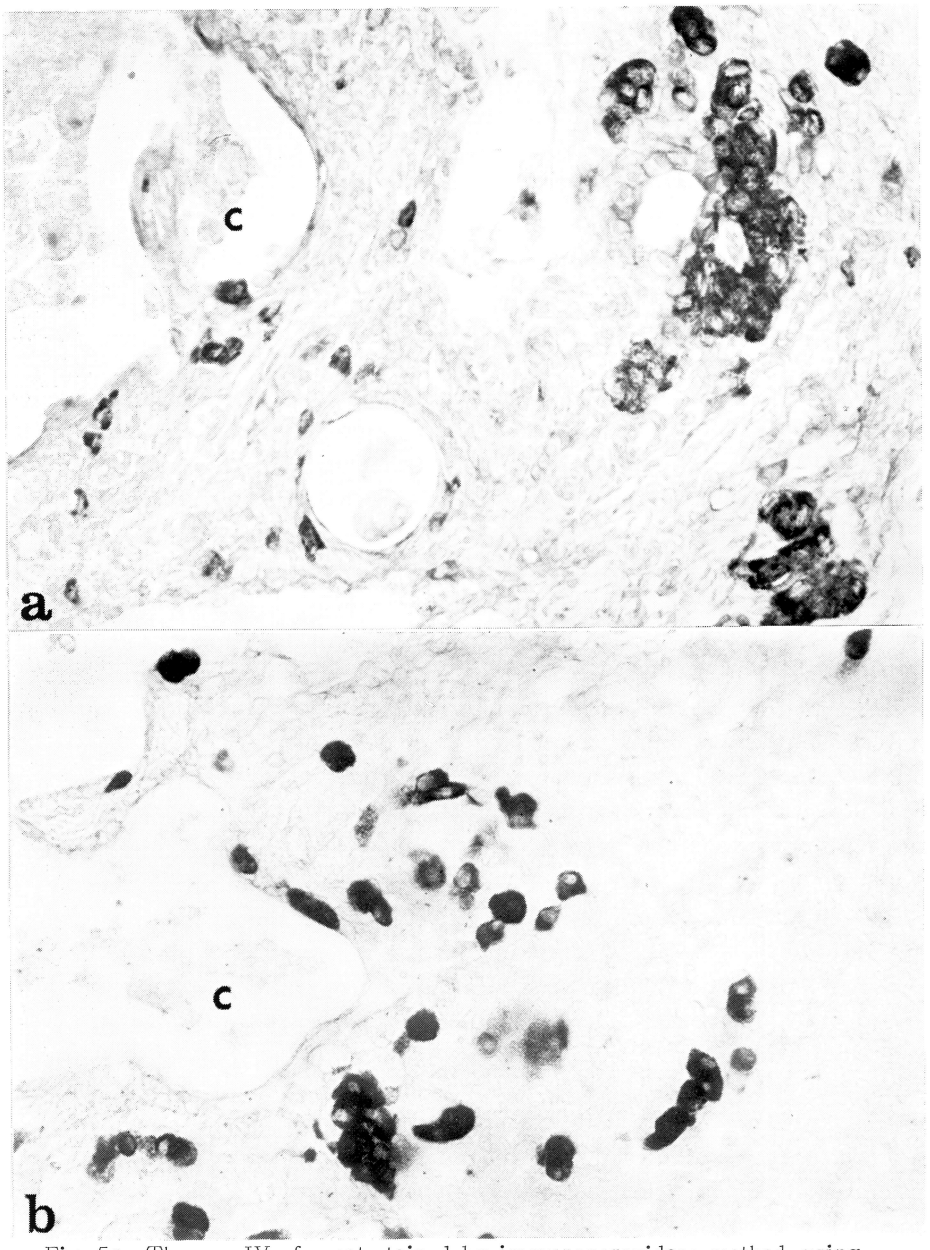


Fig. *5a*. Thymus IV of a cat stained by immunoperoxidase method using anti-porcine calcitonin antiserum. A number of C-cells filled with immunoreactive secretory granules are dispersed among thymic elements. Since the specimen was fixed in GPA solution, the background reaction is somewhat seen. C, cyst. ×290 *5b*. Thymus IV of a dog stained by immunoperoxidase method using anti-porcine calcitonin antiserum. C-cells are concentrated around cyst (C). ×290

around the cysts (Fig. 5a, b).

Thymus IV of rabbits and goats was observed in none of the thyroid lobes cut in serial sections.

Parathyroid gland III and thymus III

No C-cells were found in the parathyroid gland III and in the thymus III localized adjacent to parathyroid III in any species examined.

DISCUSSION

In accordance with the previous studies[1,2] using histological staining methods for the demonstration of C-cells, i.e., silver impregnation, pseudoisocyanin and lead-hematoxylin, the present immunocytochemical study using the specific antisera against human calcitonin, porcine calcitonin and dog C-Tg confirmed that in some mammalian species the C-cells were distributed not only in the thyroid gland but also in the parathyroid IV and thymus IV. The C-cells scattered in parathyroid IV and thymus IV were filled with numerous immunoreactive secretory granules after the immunoperoxidase stainings and revealed the same reaction intensity to these antisera as the cells in thyroids of each animal. In rabbits, cats and goats the presence of C-cells in the parathyroid IV was almost a constant feature and a great number of C-cells were widely dispersed throughout the whole parathyroid parenchyma. On the other hand, in dogs it was an occasional feature ; the frequency of occurrence was 9.8%. There were a small number of C-cells in the periphery of parathyroid parenchyma of dogs. In the thymus IV which lay adjacent to parathyroid IV, the C-cells were also observed very frequently. In dogs almost all thymus IV revealed the distribution of C-cells and in cats ten out of 12 thymus IV did.

It is well known that C-cells are derived from the ultimobranchial bodies. During early fetal periods, the ultimobranchial bodies of many mammals together with parathyroid IV and thymus IV develop from the pharyngeal pouch IV and in progressive stages join with the thyroid anlage descending from the midventral evagination of the pharynx[22,23]. Afterward, they move into thyroid parenchyma to disperse as definetive thyroid C-cells[11]. It is considered that the dispersion patterns of ultimobranchial bodies differ from species to species, and the difference is reflected in the distribution of C-cells in the thyroid, parathyroid IV and thymus IV of adult animals. In the rabbit, cat and goat the ultimobranchial bodies move slightly around the portion of contact with the thyroid, surrounding the region of parathyroid IV and also invade constantly into the parathyroid IV, while in the dog they were scattered widely throughout the

thyroid lobes but their invasion into parathyroid IV is occasional. In addition, in the rabbit, goat and other many mammalian species the ultimobranchial bodies are dispersed as solitary cells or as small clusters of cells to form thyroid C-cells, whereas in the dog and cat they are dispersed as large cell clusters. Particularly large cell groups derived from ultimobranchial bodies, which are incompletely incorporated in thyroid parenchyma and show little migration, are observed as C-cell complexes around the parathyroid IV in postnatal dogs and cats[1,2,21,24]. The C-cell complexes consist of masses of C-cells associated with other epithelial elements and various sized cysts and retain an abundance of fetal characteristcs for a very long time, showing a large number of undifferentiated cells and immatue C-cells even in adult animals.

The previous investigators[6-9] using the immunoperoxidase method with antiserum to human calcitonin failed to demonstrate the C-cells in parathyroid glands and thymus tissues of man and rats. They have emphasized that the distribution of C-cells is restricted to thyroid glands. However, these investigators did not discriminate between parathyroid IV (internal parathyroid) and parathyroid III (external parathyroid), and further between thymus IV and thymus III. Especially, in the rat there is no parathyroid IV. As clarified in the present study, C-cells are distributed in parathyroid IV and thymus IV having intimate developmental relation with the ultimobranchial body, the origin of C-cells, but not in parathyroid III and thymus III remote from the ultimobranchial body. In addition, the frequency of occurrence of C-cells in parathyroid IV is species variable ; in contrast to rabbits, cats and goats whose parathyroids IV contain almost constantly the C-cells, dogs reveal an occasional presence of the cells in them.

Calcitonin is composed of a single chain of 32 amino acids with a 1-7 intra-chain disulphide bridge at the amino terminus. In spite of having a low molecular weight, the antigenicity of calcitonin is strong. The author readily produced the antibodies against both synthetic human and native porcine calcitonin in rabbits after multiple injections of the hormone emulsified with the adjuvant without carrier proteins. There is large difference in amino acid sequence of calcitonin from various animals[25]. Therefore, the sensitive stainings with the produced antisera are limited to the C-cells of animal species synthesizing calcitonin with amino acid sequence similar to that of the antigen. The anti-human calcitonin antiserum reacted strongly to the C-cells of rabbits, but weakly to the cells of cats, goats and dogs. In many recent immunocytochemical studies for C-cells antiserum to human calcitonin has been used, and the cells of man, rats, horses, moles and deer are densely stained[7,8,26]. Anti-porcine calcitonin antiserum revealed the high immune responses to C-cells of cats,

goats and dogs, and further to the cells of mice, hamsters, guinea pigs, cows and pigs[27].

Compared with the antisera to calcitonin, the antiserum to dog C-Tg reveals a high degree of cross-reactivity to the C-cells of various mammalian species. In almost all species examined, i.e., rats, hamsters, mice, lions, cows, pigs and man in addition to cats, goats and dogs, the C-cells are densely stained by the antiserum[28], though the sensitivity of staining is somewhat different from species to species. It is considered that the structural features determining immunological specificity of various C-Tg have more numerous homology of amino acid sequence than those of calcitonin.

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