IMMUNOHISTOCHEMICAL DEMONSTRATION OF THYROID HORMONE IN PARAFFIN EMBEDDED HUMAN THYROID TISSUES*

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The immunoperoxidase technique was shown to be applicable to localization of thyroxine (T4) and triiodothyronine (T3) in 10% formalin fixed paraffin sections of human thyroid tissues including tumors. T4 was demonstrated in the colloid substance and on the luminal surface of the follicular epithelia, and less frequently in the cytoplasm of the non-neoplastic epithelia. On the contrary, cytoplasmic staining of T4 was remarkable in some thyroid tumors. The T3 localization was observed rather in the cytoplasm of the epithelial cells than in the colloid substance.

It is noteworthy that most of the thyroid neoplasms which were clinically diagnosed as non-functioning showed more or less positive staining for either T4 or T3.

The immunohistochemical technique has been effectively applied to the demonstration of peptide and protein hormones, contributing to recent progress in functional aspects of endocrine tissues and cells (6). Thyroxine (T4) and triiodothyronine (T3), however, are hormones for which the immunohistochemical method has not as yet been satisfactorily established.

Only recently Wilson et al. (11) reported the immunofluorescent method to localize the thyroid hormone in frozen sections of the rat thyroid gland. In the present study, we attempted to localize T4 and T3 in the paraffin sections of the human thyroid tissues using the immunoperoxidase technique.

MATERIALS AND METHODS

Materials. Thyroid tissue and tumors of the thyroid gland which were surgically removed from the patients with hyperthyroidism (4 cases), colloid struma (1 case) and non-functioning thyroid neoplasms (10 cases of adenoma, and 10 cases of carcinoma) were fixed in 10% buffered formalin, pH 7.2, embedded in paraffin and cut into 5 μm thick sections as usual.

Antiserum. Anti thyroxine (T4) rabbit antiserum and anti triiodothyronine (T3) rabbit antiserum were purchased from E.Y. Laboratories Inc., San Mateo.

Cal. and Cappel Laboratories, Cochranville, Pa. Since these antisera were prepared using hapten-bovine serum albumin (BSA) conjugates, the anti BSA activity of the sera were absorbed by the following procedure: the antisera diluted to twenty to forty times with phosphate buffered saline, pH 7.2 (PBS) were mixed with BSA (Albumin, bovine, Fraction V, powder, Sigma Chemical Company, St. Louis, Mo.) to a final concentration of 1% BSA, incubated at 37°C for 1 hr, left standing at 4°C overnight, and were centrifuged at 10,000 rpm for 5 min before use. The thyroid hormone absorbed antisera were prepared as follows: 10 mg of T4 (3,3', 5,5'-Tetraiodo-L-Thyronine, Sigma) or T3 (3,3',5-Triiodo-L-Thyronine Na Salt, Nakarai Chemicals, Ltd., Kyoto) was dissolved in 0.5 ml of 0.1 M sodium hydroxyde, diluted with 0.1% BSA, and was added to both anti T4 and anti T3 antisera which were previously absorbed with BSA to a final concentration of 0.1 mg hapten per ml antiserum. Thus four types of absorbed antisera, i.e. anti T4 with T4, anti T4 with T3, anti T3 with T3, and anti T3 with T4 were prepared. The peroxidase labeled anti rabbit IgG goat gamma globulin was prepared according to the method described by Nakane and Kawaoi (7).

Immune staining procedure. Deparaffinized tissue sections were rinsed in cold PBS for 10 min and reacted successively with either BSA absorbed anti T4 or anti T3 rabbit antisera at 37°C for 60 min, a peroxidase labeled second antibody at room temperature for 30 min, followed by incubation with 3,3'-diaminobenzidine with 0.005% hydrogen peroxide (3) for 5 to 10 min. For the control group the first antisera were replaced by the absorbed antisera described above. In addition, some sections were only reacted with substrate to examine the endogenous enzyme activity.

RESULTS

Most of the materials (22 of 25 cases) including relatively normal thyroid tissues, hyperplastic glands (4 cases), adenomas (9 cases) and carcinomas (9 cases) showed positive immune staining for both T4 and T3 in varieties of both intensity and distribution pattern. Non-pathologic thyroid tissues revealed weakly positive staining for T4 in the colloid substance and occasionally on the luminal surface of the follicular epithelial cells. In the hyperplastic thyroids found in the specimens from the patients of Basedow's disease an increased intensity in staining of T4 was observed as compared with those of the normal tissue, where T4 was localized in the colloid substance (Figs. 1 and 2), on the luminal surfaces of the follicular epithelia (Fig. 3), and in the cytoplasms of some epithelial cells (Figs. 2 and 3). However, there were many follicles which showed almost completely negative in the colloid substance as well as the epithelia.

The staining reactivity of anti T4 antisera which has not significantly affected by absorption with T3 was completely lost by incubation with T4 (Fig. 4). As shown in Fig. 4 no endogenous enzyme activity of the follicular epithelia was detected so far. The T3 immunoreactivity was detected in the follicular epithelial cells as well, although it was mostly undetectable in the colloid substance.

Both T4 and T3 were able to be localized in tumor tissues, where T4 was stained more frequently in adenomas than in the carcinomas. As was observed in non-
Fig. 1. Thyroid tissue with hyperplasia. The colloid substance of several follicles shows positive staining for T₄ in variable intensity. ×140

Fig. 2. Thyroid tissue with hyperplasia. T₄ is localized in the cytoplasm of some follicular epithelia as well as in the colloid substance. ×280
Fig. 3. Thyroid tissue with hyperplasia. The luminal surface of the follicular epithelia shows linear staining for T₄. Some of the epithelia also show cytoplasmic localization. ×280

Fig. 4. Thyroid tissue with hyperplasia. No positive staining was observed in the tissue reacted with anti T₄ antisera which were previously absorbed with T₄. ×140
Fig. 5. Follicular adenoma of thyroid. Localization of $T_4$ is intensively demonstrated in many epithelia as well as in the colloid substance. $\times 140$

Fig. 6. Follicular adenoma of thyroid. The tissue reacted with non-immune rabbit sera in stead of antisera showed completely negative except for the red blood cells in the blood capillaries. Note no endogenous activity of thyroid peroxidase is detected in the follicular epithelia. $\times 140$
neoplastic tissues, the colloid substance and the cytoplasm of the tumor cells lining the follicles were positive for T4 immunoreactivity with considerable variety in staining pattern (Fig. 5).

The sections which were incubated with non-immune rabbit serum in place of the antisera resulted in negative staining except for the red blood cells (Fig. 6).

The localization of T3 in tumor tissues was similar to that of T4, although the cytoplasm of the tumor cells were shown to be more intensely stained than the colloid material (Fig. 7). The T3 immune staining which was not influenced by using anti T3 absorbed with T4 decreased significantly by incubation with the anti T3 antisera absorbed with T3.

The frozen sections showed essentially the same staining patterns for T4 and T3 as those of paraffin embedded specimens, although inadequate preservation of the tissue structure frequently made identification of the positive site rather difficult.

**DISCUSSION**

The functional aspect of the thyroid tissue in normal and diseased states has been analyzed by enzyme histochemical methods (4, 5, 10) and radioautographic demonstration of the incorporated iodine substance (2). These techniques, however, demonstrate rather indirectly the states of thyroid hormone synthesis and secretion. Immunohistochemical localization of the thyroid hormone was reported for the first time by Wilson et al. (11) using the immunofluorescent technique on frozen sections of the rat thyroid gland.

In the present study, the immunoperoxidase method was successfully applied
to detect T₄ and T₃ on the surgical specimens of the human thyroid tissues which were fixed with 10% formalin and embedded in paraffin as usual. The specificity of immune staining was confirmed by the following facts: 1. Application of non-immune rabbit sera followed by peroxidase conjugate gave completely negative staining. 2. Incubation with the antisera previously absorbed with the corresponding antigens resulted in significant or complete disappearance of staining, whereas cross absorption of the antisera caused little or no effect on immune staining. 3. Incubation of the tissue in the substrate solution for peroxidase did not show any endogenous enzyme activity of the follicular epithelia, indicating that thyroid peroxidase activity (10) was inactivated in the present processing of the tissue and incubation condition.

Thus, the result of the immune staining proved that formalin fixed and paraffin embedded thyroid tissue sufficiently preserved the antigenic activity of the thyroid hormone. However, it still remains unknown how much of the initial immunoreactivity of the hormone was lost through tissue processing. A quantitative estimation of the effect on thyroid hormone antigenecity of various fixatives, incubation temperature and organic solvents should be conducted in future (8).

As Wilson et al. pointed out, the anti T₄ antisera absorbed with T₃ showed to some extent decreased staining activity as compared with the non-treated ones in the present study. Although the exact reason remained unclarified, the purity of T₃ used for absorption should be taken into consideration.

The variety in staining intensity among the individual follicles may reflect their functional state in hormone synthesis and secretion. In non-neoplastic tissues, T₄ was predominantly located in the colloid material and the luminal surface of the follicular epithelia as observed by Wilson et al. in frozen sections, but the cytoplasm of the epithelial cells revealed positive staining less frequently than the colloid material.

It seems to be noteworthy that most of the thyroid tumors which were clinically diagnosed as non-functioning disclosed positive for both or one of T₄ and T₃ by immune staining, suggesting that they should be considered as functioning in a strict sense. Further, the present study suggested that immunohistochemical detection of the thyroid hormone as well as thyroglobulin (1, 9) could be a useful technique to investigate the functional aspect of the normal and diseased thyroid glands and that application to immunoelectron microscopy would contribute to subcellular analysis of the mechanism of thyroid hormone biosynthesis and secretion.

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