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IMMUNORECOGNITION OF ANTI-SEROTONIN ANTIBODIES BY USING A RADIOLABELLED LIGAND

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Specificity of anti-serotonin antibodies to be assayed for immunocytochemical studies was tested in vitro by using a radiolabelled ligand mimicking the hapten structure. A good immunorecognition was found after coupling [^{3}H]HT* to a lysine with formaldehyde. Competition experiments by equilibrium dialysis were then carried out between [^{3}H]HT-F-ALM and both conjugated and unconjugated indoles. The cross-reactivity ratios showed that the antibody recognition, though less specific than expected, was found suitable for immunocytochemical applications.

Specific histological demonstrations of serotoninergic pathways have previously been described [1,11,12,19,23]. Antibodies have been obtained after coupling serotonin (HT) with formaldehyde to protein carriers [4,10,16,20]. However, although the immunorecognition of the antibody site had been performed with conjugated or unconjugated compounds [13,17,20], it was difficult to determine which antigenic structure the antisera would exactly recognize. In the present report, we describe a radioimmunological approach by using a radiolabelled ligand which mimicks the hapten coupled to tissues and/or to immunogens. The results obtained in vitro are compared to immunocytochemical applications.

Serotonin was coupled to bovine serum albumin (BSA) using a formaldehyde condensation reaction, according to the method of Ranadive and Sehon [16] as perfected by Grota and Brown [10]. Ten milligrams of HT-creatinine sulfate (Sigma) in 1 ml of distilled water and 1 ml of 3 M sodium acetate solution were mixed with 30 mg BSA (Sigma). The pH was adjusted to 8.5, and 1 ml of 7.5% form-

* Abbreviations: HT, serotonin; aHT, N-acetyl-serotonin; aMT, melatonin; HW, 5-hydroxytryptophan; ML, 5-methoxytryptophol; T, tryptamine; MT, 5-methoxytryptamine; ALM, N-α-acetyl-L-lysine-N-methylamide; HIAA, 5-hydroxyindole acetic acid; F, formaldehyde.

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aldehyde (Merck) added. The mixture was stirred for 5 min at room temperature. The yellow solution obtained was dialyzed against running tap water for two days at 4°C and clarified by centrifugation and filtration through a 0.45 μm Millipore filter. Spectral analysis was performed to determine the molar ratio of coupling which was found to be 45:1 (Fig. 1).

Immunization was then carried out according to the method of Vaitukaitis et al. [24] with slight modifications. Each rabbit was injected with 500 μg of immunogen emulsified in 0.75 ml of a physiological solution and 0.5 ml of complete Freund's adjuvant (Difco). Booster injections were given once a month for 4 months. Between each injection, rabbits received an intramuscular injection of the above-mentioned mixture.

As HT was principally linked to the lysine residues in immunogenic conjugates [16], we coupled [^{3}H]HT (NEN) to a lysine derivative mimicking the location of lysine within the polypeptidic chain: N-α-acetyl-L-lysine-N-methylamide (ALM; Sigma). Two hundred and fifty μl of 3 M acetate buffer, 500 μl of 0.1 M ALM solution, 30 μl of [^{3}H]HT solution were mixed (pH 8.5). Then, 500 μl of a 7.5% formaldehyde solution were added. The mixture was left for 3 min at room temperature before loading on a SPC 25 Sephadex column (1 cm × 48 cm) to separate the different compounds. The column was eluted with 2 × 150 ml of 10 mM to 0.8 M NaCl gradient in 10 mM phosphate buffer. Three elution peaks were separated. Only the last one was found to be the most immunoreactive and corresponded to the tritiated derivative [^{3}H]HT-F-ALM (binding ratio (B/T) = 0.5). The others corresponded to [^{3}H]HT free (B/T = 0.03) and [^{3}H]HT-F-HT-[H^3] (B/T = 0.13).

In view of specificity studies, non-tritiated HT and indoles close to HT were coupled to ALM by formaldehyde. Only indoles having a free amino group were

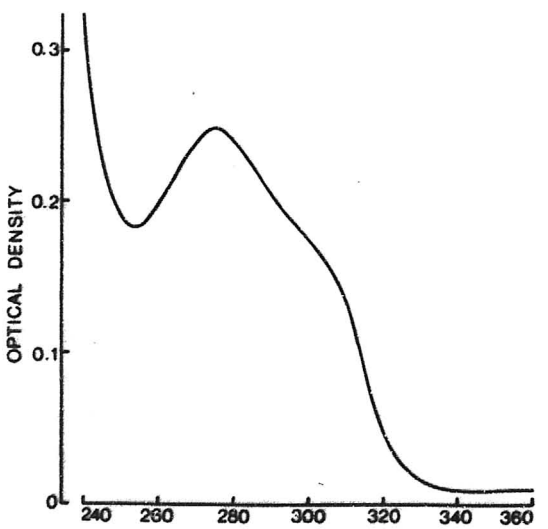

Fig. 1. Ultraviolet spectrum of HT-F-BSA. The coupling molar ratio was determined by calculating the concentration of HT and BSA at 300 nm and 280 nm, respectively. HT, 3.85×10^{-5} M; BSA, 8.6×10^{-7} M.

ALM-linked. Under these experimental conditions, the following derivatives were obtained: serotonin–formalin–ALM (HT-F-ALM), 5-methoxytryptamine–formalin–ALM (MT-F-ALM), tryptamine–formalin–ALM (T-F-ALM) and 5-hydroxytryptophan–formalin–ALM (HW-F-ALM). After being coupled, each conjugated indole was purified by chromatography through a SPC 25 Sephadex column. The same elution profile as for the tritiated derivative was obtained. As expected, melatonin (aMT), N-acetyl-serotonin (aHT) and indoles having no free amino group could not be linked to ALM.

Competition experiments were performed by radioimmunological incubations as described in [3,7,8]. The dialysis cells, fitted with large pore cellulose membranes (Sartorius SM 115 33) were rinsed with distilled water before re-use. The concentration of each conjugated indole chromatographed was determined by measuring the optical density at 280 nm. Each conjugate was mixed with an equal volume of tritiated derivative before being placed on one side of the membrane. Antibodies were diluted at 1:600 and placed on the other side of the membrane. The incubation solution then contained 0.1 M citrate buffer (pH 6.2), 1 mg/ml of BSA and 10 mM sodium azide. The final volume (2 × 150 μl) was dialyzed for 20 h at 4°C. After this incubation time, 100 μl were taken from each side of the membrane and put into vials containing 1 ml of Instagel. Beta radioactivity was evaluated and the binding ratio (B/T) calculated as follows: bound radioactivity (B) (obtained by deducting free radioactivity from total radioactivity) divided by total radioactivity (T).

First, competition experiments were established at equilibrium dialysis between [^{3}H]HT-F-ALM and non-conjugated indoles (Fig. 2A). Except for HIAA, no displacement was observed for uncoupled compounds and even HT was barely

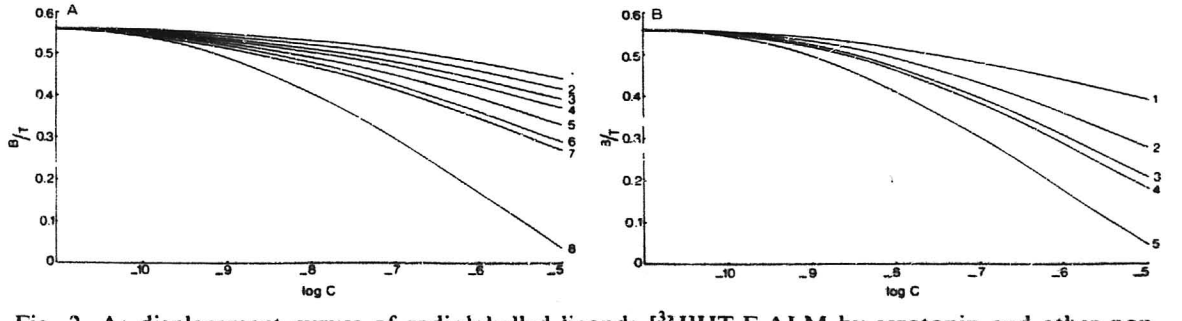

Fig. 2. A: displacement curves of radiolabelled ligand: [^{3}H]HT-F-ALM by serotonin and other non-coupled indoles: (1) 5-methoxytryptamine, N-acetylserotonin; (2) 5-methoxytryptophol; (3) melatonin; (4) 5-hydroxytryptophan; (5) tryptamine; (6) serotonin; (7) 5-hydroxyindole acetic acid; and (8) serotonin–formalin–N-α-acetyl-L-lysine-N-methylamide. For radioimmunological incubations, anti-HT antibodies were used at a 1:600 dilution. B: displacement curves of [^{3}H]HT-F-ALM by indole derivatives coupled to ALM by formaldehyde: (1) N-acetylserotonin-F-ALM (aHT-F-ALM); (2) 5-hydroxytryptophan-F-ALM (HW-F-ALM); (3) tryptamine-F-ALM (T-F-ALM); (4) 5-methoxytryptamine-F-ALM (MT-F-ALM); and (5) serotonin-F-ALM (HT-F-ALM). The most immunoreactive was MT-F-ALM at a factor of 8 from HT-F-ALM. The other compounds followed in this order: T-F-ALM at 13 from HT-F-ALM, HW-F-ALM at 69 from HT-F-ALM. For aHT-F-ALM, no immunoreactivity was found.

recognized. On the other hand, the antibody affinity for [^{3}H]HT was 140 times less compared to the one observed for [^{3}H]HT-F-ALM. The difference observed between [^{3}H]5-HT and [^{3}H]HT-F-ALM underlined the importance of formaldehyde residues. At this point, adsorption tests using free-HT or any other uncoupled compound were not applicable to tests using formaldehyde-coupled compounds. Second, displacement curves were established between [^{3}H]HT-F-ALM and indole derivatives (Fig. 2B) which corresponded to the indole fixation to the tissues. The resulting cross-reactivity ratio was calculated as follows: HT-F-ALM concentration upon each conjugated or non-conjugated derivative at half-displacement (see Table I). The other conjugates exhibited an immunoreactivity of variable intensity: MT-F-ALM and T-F-ALM had cross-reactivity ratios of 1:8 and 1:13, respectively; while for other compounds they were as follows: HW-F-ALM, 1:69; non-coupled HT, 1:140; and T, 1:525.

For the immunocytochemical localization of HT, 200 g male Wistar rats were perfused intracardially with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.5. Fifty micrometer Vibratome (Oxford) sections were incubated with anti-HT antibodies diluted at 1:750. Subsequently the bound antisera were visualized by the peroxidase–anti-peroxidase technique [21] with slight modifications as in refs. 2 and 15. Staining appeared in the region of the dorsal raphe nucleus [19] and in other areas where HT-containing cell bodies are reported to be located. The extensive HT fiber network in the central nervous system was also stained (Fig. 3). The antibody was pre-adsorbed on HT-F-haemoglobin, synthesized as the immunogen. When the concentration of conjugated-HT in HT-F-haemoglobin was 10^{-6} M, the staining disappeared. This test confirms the validity of HT localization after coupling by formaldehyde.

As stated in the introduction, it was possible to determine the antibody specificity by using a radioimmunological assay. Displacement curves were established from competition experiments between [^{3}H]HT-F-ALM and conjugated or unconjugated indoles coupled by formaldehyde to lysine. Under these experimental conditions,

TABLE I

CROSS-REACTIVITY RATIOS OF HT-F-ALM, INDOLES AND DERIVATIVES

Compounds	Ratio[a]
Serotonin-F-ALM (HT-F-ALM)	1
5-Methoxytryptamine-F-ALM (MT-F-ALM)	1:8
Tryptamine-F-ALM (T-F-ALM)	1:13
5-Hydroxyindole acetic acid	1:51
5-Hydroxytryptophan-F-ALM- (HW-F-ALM)	1:69
Serotonin (HT)	1:140
Tryptamine (T)	1:525

[a]HT-F-ALM concentration divided by the coupled or non-coupled indole concentration at half displacement.

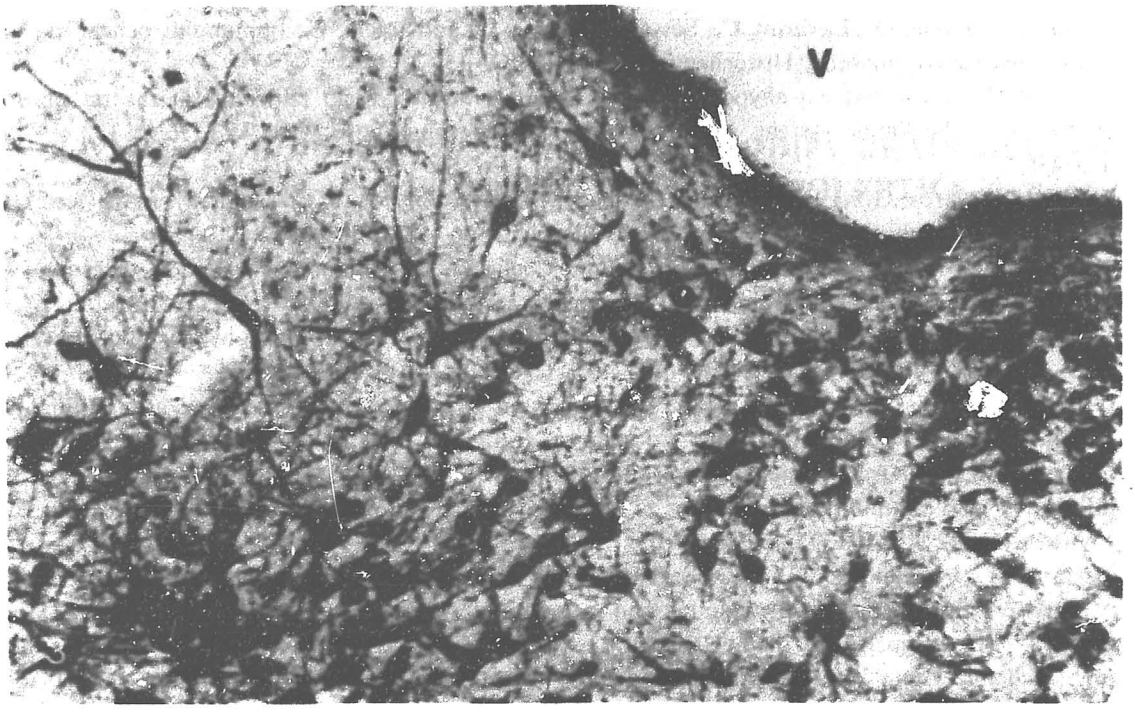

Fig. 3. Transversal 50 μm Vibratome sections of a treated formalin fixed rat brain in the region of the dorsal raphe nucleus. V, ventricule. Bar = 200 μm. Sections were incubated with HT-antiserum at 1:750. This figure shows serotonin-containing cell bodies in the dorsal raphe nucleus. Note the presence of very thin fibers also on the ependymal layer.

conjugated-HT was better recognized than non-conjugated HT by a factor 140. Specificity tests were carried out with amines coupled with formaldehyde, either to a gelatin gel [17], to a protein-carrier [14,22] or to an amino acid containing a free amino group (e.g. ALM). These results were in agreement with those described in the literature [3,5–8,18] which took into account the recognition by the antibody site of three equivalent amino acid residues [9]. Though serotoninergic detection in tissues appeared to be specific and in close agreement with the literature, cross-reactivity ratios between HT-F-ALM and MT-F-ALM on the one hand, and between HT-F-ALM and T-F-ALM on the other, were smaller than those obtained with antibodies raised against small-sized molecules [6–9,18]. At this point, the antibody site depends on the structure of HT–formaldehyde residue–lysine and to a lesser extent on the proteic environment. The discrepancy between the good immunocytochemical specificity and the poorer results obtained from RIA has been tested. This could result from the poor fixation by formaldehyde of both MT and T to the tissues (personal results).

The cross-reactivity noticed between coupled HT and conjugated indoles, led us to take into account the limitations of formaldehyde coupling and to prefer the use of glutaraldehyde. The results obtained by using this coupling agent are to be published soon in another article.

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