A Neuroendocrine Marker In Tissues of the Immune System

Abstract. *Antibodies to chromogranin, a secretory protein marker for the diffuse neuroendocrine system, were used to analyze rat lymphoreticular tissues by means of immunochemistry and immunohistochemistry. Chromogranin-positive cells were present in spleen, lymph node, thymus, and fetal liver. When these organs were gently dispersed and separated on a Ficoll gradient, the chromogranin-immunoreactive cells became enriched in the dense red-cell pellets. The unexpected distribution of these neuroendocrine cells in all immunologically relevant structures suggests that they may link the nervous and immunological systems.*

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The diffuse neuroendocrine system (DNS) or amine precursor uptake and decarboxylation (APUD) system comprises a family of cells having features common to both neurons and endocrine cells (*1*). Among its members are adrenal chromaffin cells, anterior pituitary cells, gut enterochromaffin cells, pancreatic islet cells, thyroid calcitonin-producing parafollicular cells, and parathyroid cells. These cells have homeostatic regulatory roles because of both their anatomical location and their secretion of diverse biologically active peptides. The protein chromogranin is an accurate marker for the diffuse neuroendocrine system (*2*). Although much is known about the structural properties of chromogranin, little is yet known about its function (*3*). Combined immunochemical and immunohistochemical studies show that chromogranin is present in neurons as well as in the DNS (*2*, *4*). Furthermore, chromogranin can be detected in the thymus epithelial cells, which have been included as part of the DNS. In the normal immune system, these thymic constituents secrete polypeptides, the thymosins, which are immunoregulatory agents (*5*).

While studying antibodies to chromogranin in the rat, we examined the spleen as a control tissue. Immunohistochemical staining revealed in the spleen a cell with neuroendocrine properties. Immunoperoxidase staining (Fig. 1A) shows chromogranin-positive cells in the 5-day rat spleen. Although the positive cells are distributed throughout the spleen, they seem to be selectively concentrated in subcapsular areas. The adult spleen differs from the neonatal spleen both morphologically and with respect to the chromogranin-containing cells. Whereas the 5-day spleen exhibits little of the characteristic splenic morphology, in the 4-month spleen the white and red pulp are distinct. The chromogranin-immunoreactive cells are not evenly distributed throughout the adult spleen, but seem to be excluded from Malpighian (white pulp and lymphoid) zones. The splenic cells that contain chromogranin are round to ovoid, large, and not representative of autonomic termini or dendrites.

Having observed the chromogranin-containing cells in the spleen, we also examined other lymphoreticular tissues. Of particular interest are the lymph nodes and thymus. Lymph nodes contain positive, sparsely distributed cells (Fig. 1B), most of which are scattered in the interfollicular and subcapsular areas of the cortex without any apparent concentration in follicular or perifollicular areas. However, chromogranin-positive cells are also located in the medullary cords. Higher magnification of the lymph node (Fig. 1D) reveals large chromogranin-positive cells with abundant cytoplasm. The positive spleen cells were morphologically similar. In the thymus, epithelial cells and another scattered, polygonal, nonepithelial subpopulation of cells were detected.

Bone marrow is another heterogeneous tissue containing lymphoreticular elements. Embryologically, the bone marrow constituents are derived from cells that have migrated from fetal liver, yolk sack, and diffuse mesodermal areas. In view of its accessibility and ease of processing, as well as its content of hematopoietic and histiocytic cells, fetal livers from several stages of development were examined for chromogranin. Fetal (19-day) liver contains sparse, but strongly chromogranin-positive cells that tend to be distributed in the sinusoids around the central veins (Fig. 1C). The chromogranin-containing cells in the fetal liver are morphologically similar to those in the spleen and lymph node. These cells in fetal liver may be important to the immune system since they do not seem to be inherent hepatic constituents. Rather, they seem to emigrate from the liver during development. In the adult rat the hepatic morphology of radiating cords of cells becomes clearer as hematopoietic elements decrease. Chromogranin-containing cells could not be found in adult rat livers devoid of hematopoietic elements.

To confirm that our antisera and monoclonal antibodies were detecting the chromogranin molecule in these tis-

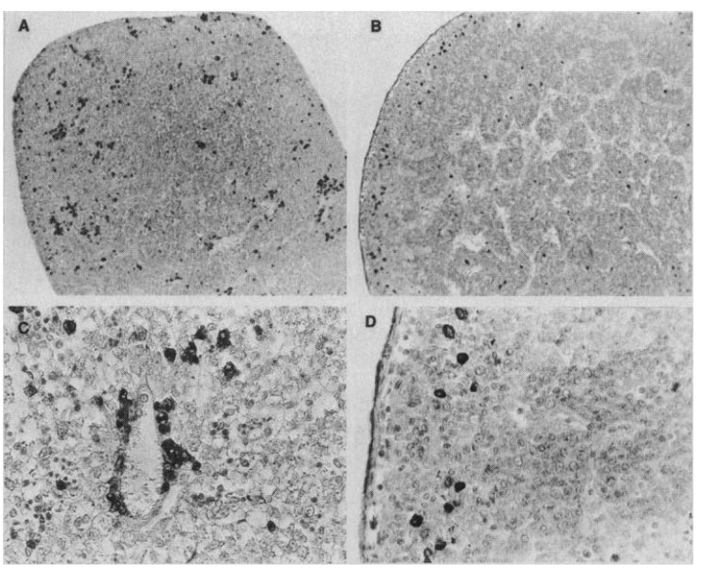


Fig. 1. Immunohistochemical analysis of rat lymphoreticular tissues. Tissues were fixed for 1 hour at room temperature in Bouin's fixative. The tissues were rinsed in 10 m*M* potassium phosphate containing 0.15*M* NaCl before being embedded in paraffin. Sections (6 μm) were incubated on chromium-aluminium–coated slides overnight at 4°C with rabbit antibody to bovine chromogranin (*3*), diluted at a ratio of 1:200. The slides were then developed with the peroxidase-antiperoxidase procedure of Sternberger (*8*), with diaminobenzidine used as the final substrate. Similar reactions were given with monoclonal antibodies to chromogranin. The antibodies and antisera were prepared as described in (*3*); that is, all antigens were denatured with SDS and purified from unstained electrophoretic gels as a final step. (A) Five-day spleen, ×35; (B) 2-week lymph node, ×70; (C) 19-day fetal liver, ×140; (D) 2-week lymph node, ×140.

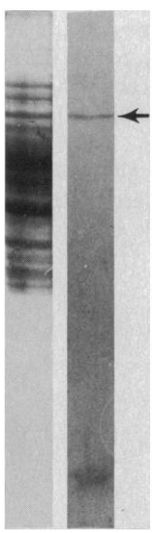
sues, we performed immunoblot analysis (*6*).

The chromogranins are a family of polypeptides of which the most abundant species has a relative molecular weight of 75,000, although species of both higher and lower molecular weight can be detected. The stained gel and immunoblot of a homogenized rat lymph node show the predominant chromogranin band (Fig. 2). Similar results were seen with homogenates of both spleen tissue and 19-day fetal liver.

In attempting to isolate the chromogranin-containing cells from whole tissues, we removed spleens, lymph nodes, fetal livers, or thymuses, dispersed the cells in isotonic media containing 1 percent bovine serum albumin, and layered them on a cushion of Ficoll (1.077 g/ml). This classical procedure separates the mononuclear cells, or "buffy" coat, as a layer above the Ficoll, while the erythrocytes and some denser leukocytes sediment as a pellet. The chromogranin-positive cells were enriched in the erythrocyte pellet. To confirm this selective cellular concentration in spleen, we analyzed these cell fractions by immunoblots. Figure 3 shows the Coomassie blue–stained sodium dodecyl sulfate (SDS) polyacrylamide gels of four fractions from the Ficoll gradients, as well as the immunostaining developed with antiserum to chromogranin on an immunoblot of an identical gel. Only the erythrocyte pellet was enriched in cells that contained chromogranin polypeptides. In similarly analyzed lymph nodes and fetal liver, chromogranin-containing cells were again found only in the denser, red-cell pellet. This property places the chromogranin-containing element among a class of cells with higher specific gravity than the lymphocytes, monocytes, macrophages, and plasma cells that remain above the 1.077-g/ml liquid. Morphologically they are not polymorphonuclear leukocytes, eosinophils, or basophils. Immunoperoxidase staining of adjacent sections of neonatal spleen for chromogranin and class II (Ia) antigen expression demonstrated that the distribution of chromogranin-containing cells was markedly different from that of Ia-positive cells. Thus, the cells positive for the neuroendocrine marker are neither typical antigen-presenting cells nor plasma cells. We do not know whether the cells are stromal elements or a subgroup of the migratory cells that pass through these organs.

The interactions of the nervous and immune systems form the basis of a field termed "neuroimmunomodulation" (*7*). Endocrine components of these interactions have been identified, among them the pituitary, the hypothalamus, and the thymus epithelium. However, no common biochemical "thread" has been found through these diverse tissues. Such a marker could potentially be a common standard in natural and experimental perturbations of the nervous and immunological systems. Using a recently established marker for the DNS we have identified cells in the spleen, lymph node, thymus, and fetal liver that may form a part of this regulatory network. In addition to identifying these cells by immunohistochemistry, we have demonstrated the enrichment of cells containing chromogranin polypeptides by cell fractionation techniques.

Several lines of investigation must be pursued to establish these neuroendocrine cells as significant components in neuroimmunomodulatory interactions. The presence or absence of known immunological cell surface markers is being studied. The ultrastructural and in vitro immunological properties of the cells will be examined. Furthermore, the secretory nature of these cells must be proved and their secretory products isolated and identified.



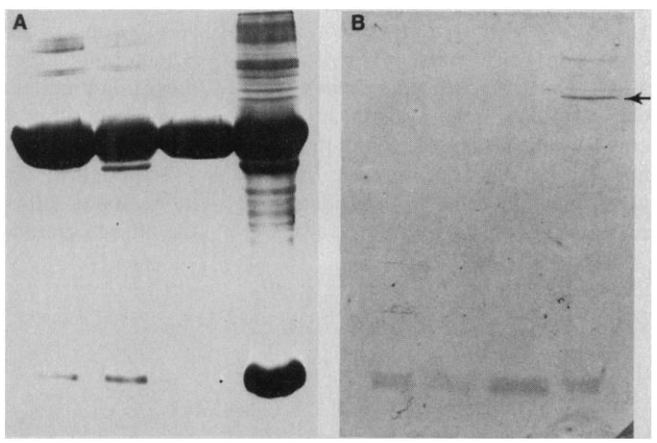


Fig. 2 (left). Immunoblot analysis of lymphoreticular tissue. Four cervical lymph nodes were removed from a freshly killed 3-week-old rat and homogenized in 0.5 ml of distilled water. The homogenate was centrifuged in a 1.5-ml microcentrifuge tube for 2 minutes. To the supernatant was added tenfold concentrated SDS sample buffer (*9*). An 8-μl sample was run on two lanes of a Hoefer minigel, 10 percent in polyacrylamide concentration. One lane of the gel was stained with 1 percent Coomassie blue in 7 percent acetic acid containing 40 percent methanol, and the other was electrophoretically transferred to nitrocellulose paper (*6*). The paper was then blocked with bovine serum and incubated overnight with antiserum to chromogranin diluted to a ratio of 1:100. The paper was then washed, incubated with peroxidase-conjugated goat antibody to rabbit immunoglobulin G and then developed with 4-chloro-1-naphthol. The left strip is the stained gel and the right strip is the immunoblot. The arrow shows the position of the 75,000-dalton chromogranin species. Fig. 3 (right). Fractionation of spleen on Ficoll gradient. A single 2-week-old rat spleen was dispersed in Hanks balanced salt solution containing 0.1 percent bovine serum albumin by being minced with scissors and passed through a 60-mesh screen. After being washed once with 10 ml of this solution, the cells were resuspended in 5 ml of the Hanks-albumin buffer and layered above 2 ml of Ficoll-Hypaque (density, 1.077 g/ml) in a 15-ml centrifuge tube (Falcon). After centrifugation for 20 minutes at 1500 rev/min in a refrigerated tabletop centrifuge (Beckman L4) at 4°C, four layers were removed: (i) the top, buffer layer; (ii) the leukocyte, or "buffy layer;" (iii) the Ficoll solution layer; and (iv) the pellet, or erythrocyte layer. Each layer was diluted with 10 ml of the Hanks-albumin solution and centrifuged again. The cell pellets were then each homogenized in 0.5 ml of distilled water and prepared for electrophoresis. Again, an 8-μl sample was loaded in each lane. Identical gels were run to obtain (A) gels stained with Coomassie blue as well as (B) immunoblots. In each, the samples are (i) to (iv), from left to right. Although the amount of protein in each lane is different, the same proportion of a single rat spleen is represented. Thus, the chromogranin-positive cells are enriched in the pellet fraction. The arrow shows the position of migration of the 75,000-dalton chromogranin.

References and Notes

1. T. Fujita and S. Kobayashi, *Trends Neurosci.* **2**, 27 (1979); A. G. E. Pearse, *Mikroskopie* **36**, 257 (1980).
2. D. T. O'Connor, D. Burton, L. J. Deftos, *Life Sci.* **33**, 1657 (1983); B. S. Wilson and R. V. Lloyd, *Am. J. Pathol.* **115**, 458 (1984); J. A. Nolan, J. Q. Trojanowski, R. Hogue Angeletti, *J. Histochem. Cytochem.*, in press.
3. J. Settleman, J. Nolan, R. Hogue Angeletti, *J. Biol. Chem.* **260**, 1641 (1985); J. Settleman, J. Nolan, R. Fonseca, R. Hogue Angeletti, *ibid.*, p. 1645.
4. P. Somogyi *et al.*, *Brain Res. Rev.* **8**, 193 (1984).
5. K. K. Oates and A. L. Goldstein, *Trends Pharm. Sci.* **5**, 347 (1984).
6. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979).
7. N. H. Spector, in *Immunoregulation*, N. Fabris *et al.*, Eds. (Plenum, New York, 1983).
8. L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, ed. 2, 1979).
9. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).

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