Immunoreactivity of Hormonally-Characterized Human Endocrine Cells against Three Novel Anti-Human Chromogranin B (B11 and B13) and Chromogranin A (A11) Monoclonal Antibodies*

Roberto Buffa1, Ambrogio Gini1, Micaela Pelagi2, Antonio G. Siccardi2, Chiara Bisiani3, Antonia Zanini3 and Enrico Solcia1

Department of Human Pathology and Genetics1, University of Pavia, Pavia; Department of Medical Biology and Genetics2, University of Milan; and CNR Cytopharmacology Center3, Milan, Italy

Summary. Two novel monoclonal antibodies, called B11 and B13, directed exclusively against human chromogranin B (CgB) and another antibody, A11, specific for human chromogranin A (CgA), were obtained by immunization of mice with chromaffin granules, the fusion of their splenocytes, the screening of hybridomas supernatants by ELISA and immunohistochemistry, and characterization of the antibodies by two-dimensional immunoblotting.

The antibodies were used in immunohistochemical tests to investigate the distribution of CgA and CgB in hormonally-identified cells of the human endocrine system. The A11 antibody confirmed the occurrence of CgA in gut EC, ECL, gastrin, secretin and neurotensin cells, pancreatic A and PP cells, parathyroid chief cells, pituitary TSH and gonadotrope cells and adrenal medullary cells. Only a fraction of CgA-immunoreactive cells in the human gut and pancreas showed C-terminus arginine-glycinamide immunoreactivity, suggesting pancreastatin storage. Both CgB antibodies showed immunoreactivity in gastrin cells, intestinal (but not gastric) EC cells, pancreatic A and PP cells, pituitary TSH and gonadotrope cells and adrenal medullary cells. In addition, the B11 antibody stained thyroid C cells and the B13 antibody stained the Golgi area of pituitary GH cells. It is concluded that most CgB is stored in the same cells showing CgA, although some CgA-rich cells, like gastric EC and ECL cells, lacked B11 and B13 immunoreactivities and some CgA-poor cells, like human thyroid C cells, showed intense B11 immunostaining.

Chromogranin A (CgA), chromogranin B/secreto-granin I (CgB) and chromogranin C/secreto-granin II (Sg II) belong to a family of anionic proteins known to be concentrated in secretory granules of most amine/peptide producing endocrine cells as well as in neuronal tissues (Fischer-Colbrie and Frischenschlager, 1985; Rosa et al., 1985; Benedum et al., 1986, 1987). Two peptides isolated from the pancreas, pancreastatin (Tatemoto et al., 1986) and β-granin (Hutton et al., 1987), have been shown to derive from CgA (Konecki et al., 1987); and two other peptides of pituitary origin, called GAWK and CCB, derive from CgB through processing at paired basic residues (Benedum et al., 1987; Benjannet et al., 1987). In addition, a 67KD anionic protein (and a related 35/32 KD dimer in reducing conditions) has been detected in secretory granules and Golgi cisternae/vesicles of many endocrine cells (Krisch et al., 1988).

All these secretory proteins and related peptides have proved to be useful markers of endocrine cells and tissues as well as of related endocrine growths (O'Connor et al., 1983; Wilson and Lloyd, 1984; Hagn et al., 1986; Rindi et al., 1986; Solcia et al., 1986; Lloyd et al., 1988). However, the distribution of these proteins and peptides resulting from immunohistochemical studies has been shown to vary according to animal species investigated, antibodies/antiserum used, type of tissue fixation and processing employed (Grube et al., 1986; Hagn et al., 1986; Rindi et al., 1986; Lloyd et al., 1988).

*This investigation was supported in part by grants from the Italian Consiglio Nazional delle Ricerche (Special Projects “Biomedical Engineering” and “Oncology”).
To ensure reproducibility and easy interpretation of immunohistochemical findings on human pathologic tissues, well characterized monoclonal antibodies with known reactivity against the various molecular forms and fragments of these partly homologous proteins, showing potential sites of antibody crossreactivity, are needed. The full reactivity of antibodies in pathologic specimens routinely fixed in aldehyde solutions and embedded in paraffin is better obtained with antibodies directed against human antigens. Unfortunately, only monoclonal antibodies directed against human CgA (LLOYD and WILSON, 1983; LLOYD et al., 1988) and the 67KD protein (KRISCH et al., 1988) are presently available. To our knowledge, no antibodies directed against human CgB or SGII are available, although antisera against the two CgB derived peptides GAWK and CCB have been obtained (BENJANNET et al., 1987; BISHOP et al., 1988).

In the present paper three novel monoclonal antibodies directed against human chromogranin A (A11) and chromogranin B (B11 and B13) have been used to localize these proteins in human endocrine tissues and hormone-identified cell types.

MATERIALS AND METHODS

Antibodies
Antibodies directed against human chromogranins/secretogranins were obtained by immunizing mice with whole chromaffin granules isolated from human pheochromocytoma (PELAGI et al., 1988). Fifty hybridoma supernatants obtained from the splenocytes of hyperimmune mice were analyzed by ELISA, immunocytochemistry and two-dimensional immunoblotting. At least eight different patterns of immunoreactivity were obtained against various molecular forms of CgA, CgB and unknown reactants. One hybridoma exclusively directed against CgA (A11) and two directed against CgB (B11 and B13) were subcloned. In two dimensional immunoblotting tests, the A11 monoclonal stained the same molecular species of human CgA shown by the LK2H10 antibody of LLOYD and WILSON (1983), with an additional low Mr spot only recognized by our antibody. The B11 antibody recognized two major forms of human CgB and several degradation products, while the B13 antibody recognized only the highest molecular forms (PELAGI et al., 1988).

A rabbit antiserum (i665/002) directed against the C-terminal part of arginin-vasopressin (AVP) was purchased from UCB Bioproducts (Brussels). In spot tests on paper (BUFFA et al., 1982) and immunabsorp-

<table>
<thead>
<tr>
<th>Cell</th>
<th>A11</th>
<th>B11</th>
<th>B13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid, C cell</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Parathyroid, chief cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pancreas, A cell</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>B</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stomach, EC cell</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ECL</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intestine, EC cell</td>
<td>+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>C-t. gastrin</td>
<td>+</td>
<td>-/+</td>
<td>+/</td>
</tr>
<tr>
<td>secretin</td>
<td>+</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>GIP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>motilin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NT</td>
<td>+/</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>GLI/PYY</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pituitary, GH cell</td>
<td>-/+</td>
<td>-</td>
<td>+ (1)</td>
</tr>
<tr>
<td>PRL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ACTH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TSH</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FSH/LH</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adrenals, A cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ : reactive; ± : faintly reactive
- : non-reactive
+/− : some cells reactive, other cells unreactive
(1) : Golgi area only; ND : not done
In 0.15 M Tris buffered saline (TBS) pH 7.6 supplemented with 10% human pooled serum and 1% human albumin. The immunoreactivity was revealed by the avidin-biotin-peroxidase procedure (Rindi et al., 1986; Pelagi et al., 1988). Controls included the use of non-immune Igs and sera, and that of antibodies or antisera directed against antigens different from the ones under study. Double immunolabeling of the same section with Cg antibodies and anti-hormone sera was performed as previously described (Buffa et al., 1987).

RESULTS

The results obtained with A11, B11 and B13 monoclonal antibodies are presented in Table 1 and Figures 1-9.

Cells reacting with the i665/002 serum directed against C-terminus arginine-glycinamide were detected in the human small intestine, pancreas and pyloric mucosa (Fig. 10). In double immunostaining tests they corresponded to a fraction of chromogranin A (A11)-immunoreactive cells, some of which showed C-terminus PP-immunoreactivity (PP-221 serum; Fiocca et al., 1983, 1987). In serum preabsorption tests, AVP did not hinder immunostaining with PP-221 serum, while it did prevent immunostaining with i665/002 serum. Conversely, the C-terminus PP31-36 sequence, which prevented immunostaining with PP-221 serum, failed to prevent immunoreactivity with i665/002 serum.

DISCUSSION

The three novel anti-human chromogranins antibodies proved effective in detecting endocrine cells in routinely processed paraffin-sections of normal human tissue.

The A11 anti-chromogranin A monoclonal, which in immunoblotting tests detected the same molecular species recognized by the LK2HID antibody, with the only addition of one low Mr spot (Pelagi et al., 1988), also stained essentially the same cells in immunohistochemical tests (Wilson and Lloyd, 1984; Rindi et al., 1986; Solcia et al., 1986; Lloyd et al., 1988) as shown by the latter antibody.

The two novel anti-chromogranin B monoclonals B11 and B13 confirmed the occurrence of CgB in human pancreatic A cells, pyloric G cells, adrenal A and NA cells and pituitary gonadotrope or TSH cells, as already shown in corresponding cells of various mammals by using an anti-bovine CgB serum (Hagn et al., 1986; Rindi et al., 1986; Buffa et al., 1987).

---

**Fig. 1.** GH cell-rich area of the human-pituitary incubated with B13 CgB antibody. Note immunoreactive spots in the Golgi area of GH cells and cytoplasmic staining of a cell in the center of the micrograph. Immunoperoxidase. ×450

**Fig. 2.** CgB immunostained (TSH+gonadotrope) cells of the human pituitary. B11 antibody, immunoperoxidase. ×450

**Fig. 3.** Intense CgB immunoreactivity of human thyroid C cells. B11 antibody, immunoperoxidase. Nomarski optic. ×450
Fig. 4. CgB immunostaining with B11 antibody (a) is localized partly to glucagon-immunoreactive cells (b) of a human pancreatic islet. Immunoperoxidase. ×350

Fig. 5. CgB immunoreactivity with B11 antibody (a) localized to PP-immunoreactive cells (b) of the human pancreas. Immunoperoxidase. ×350

Figs. 6 and 7. CgB immunoreactivity with B11 antibody of pyloric (6a) and duodenal mucosa (7a) is localized to C-terminal gastrin immunoreactive cells (6b and 7b). Immunoperoxidase. ×250

Figs. 8 and 9. CgB immunoreactivity with B13 antibody of pyloric (8a) and duodenal mucosa (9a) is localized to C-terminal gastrin immunoreactive cells (8b and 9b). Note in 9b a reactive cell lacking CgB reactivity in 9a, possibly corresponding to a cholecystokinin cell. Immunoperoxidase. ×250
In addition CgB has been localized in human thyroid C cells and parathyroid chief cells, thus confirming previous results by Lloyd and coworkers (1988) using Winkler's anti-bovine CgB antiserum. Using the same antiserum, negative results (Hagn et al., 1986) have been obtained in human parathyroid glands.

The two anti-CgB monoclonals, although giving the same results for most endocrine cells, showed a distinctive reactivity in thyroid C cells, positive with B11 and negative with B13 antibody, and in pituitary GH cells, positive with B13 and negative with B11 antibody. This was not unexpected, given the different reactivity of the two antibodies toward the smaller molecular species of human CgB in immunoblotting tests (Pelagi et al., 1988).

Present results confirm in man the largely overlapping distribution of chromogran A and B among amine/peptide producing endocrine cells which has been already noted in various animal species using antisera directed against bovine chromogranins (Hagn et al., 1986; Rindi et al., 1986; Buffa et al., 1988; Lloyd et al., 1988). Pancreatic glucagon and PP cells, pituitary gonadotrope and TSH cells, parathyroid chief cells and adrenal medullary cells are among those human cells showing consistent CgA and CgB co-localization. However, an apparent tendency of the two chromogranins to segregate is shown by thyroid C cells and several gut endocrine cells, with a prevalence of CgB in calcitonin and gastrin cells, and of CgA in argentaffin EC cells and argyrophil ECL cells.

Tissue and cell specific posttranslational processing of chromogranin molecules, with resulting changes in antibody reactivity, may account in part for these results. In fact, antisera directed against GAWK peptide, a fragment of CgB, stain human gastric EC cells (Bishop et al., 1988) despite their non-reactivity with our CgB antibodies. The fact that only a relatively minor fraction of CgA-immunoreactive cells react with an antiserum recognizing the C-terminal arginine-glycineamide sequence present in pancreastatin molecule (but not in CgA molecule) as well as the selective distribution of β-granin in respect to CgA (Hutton et al., 1988) also suggests that chromogranin molecules may undergo tissue and cell specific posttranslational processing. In particular, only a part of CgA should serve as pancreastatin precursor.

This tissue/cell specific posttranslational processing of chromogranins/secertogranins molecules, as well as their selective distribution or colocalization, might offer a versatile and convenient mechanism for controlling the intragranular posttranslational processing of hormonal peptides by interaction of chromogranins' anionic sites with the basic amino-acids involved in prohormones cleavage (Rindi et al., 1986). In fact, chromogranin A has been shown to act as a reversible inhibitor of a serine protease cleaving several prohormones into their biologically active products (Seidah et al., 1987).

The distribution of both LK2H10 and A11 immunoreactivity in human endocrine cells and tumors closely parallels the distribution of Grimelius' silver reactivity in the same structures. This further supports the hypothesis that CgA anionic sites, possibly in conjunction with prohormone cationic groups, may account for the Ag⁺ complexing sites responsible for the argyrophilia of endocrine granules (Solcia et al., 1976, 1986; Varnedel et al., 1985; Rindi et al., 1986). The contribution of CgB to Grimelius' argyrophilia remains difficult to ascertain. The failure of heavily argyrophil gastric EC and ECL cells to react with our B11 and B21 anti-CgB monoclonals is balanced by the reactivity of the same cells with antibodies directed against GAWK, a fragment of human CgB (Bishop et al., 1988).

The abundant anionic residues of chromogranins/secertogranins and related molecules, like the 67KD protein defined by the HISL-19 antibody (Krisch et al., 1988), are likely involved in the mechanisms known to provide selective detection of secretory granules in endocrine cells, as masked basophilia or metachromasia (Solcia et al., 1968) and lead-hematoxylin (Solcia et al., 1969), as well as in the intra-
Immunoreactivity of Hormonally-Characterized Human Endocrine Cells against Three Novel Anti-Human Chromogranin B (B11 and B13) and Chromogranin A (A11) Monoclonal Antibodies*

Roberto Buffa1, Ambrogio Ginì, Micaela Pelagì, Antonio G. Siccardi2, Chiara Bisiani3, Antonia Zanini3 and Enrico Solcia1

Department of Human Pathology and Genetics1, University of Pavia, Pavia; Department of Medical Biology and Genetics2, University of Milan; and CNR Cytopharmacology Center3, Milan, Italy

Summary. Two novel monoclonal antibodies, called B11 and B13, directed exclusively against human chromogranin B (CgB) and another antibody, A11, specific for human chromogranin A (CgA), were obtained by immunization of mice with chromaffin granules, the fusion of their splenocytes, the screening of hybridomas supernatants by ELISA and immunohistochemistry, and characterization of the antibodies by two-dimensional immunoblotting.

The antibodies were used in immunohistochemical tests to investigate the distribution of CgA and CgB in hormonally-identified cells of the human endocrine system. The A11 antibody confirmed the occurrence of CgA in gut EC, ECL, gastrin, secretin and neurotensin cells, pancreatic A and PP cells, parathyroid chief cells, pituitary TSH and gonadotrope cells and adrenal medullary cells. Only a fraction of CgA-immunoreactive cells in the human gut and pancreas showed C-terminus arginine-glycinamide immunoreactivity, suggesting pancreastatin storage. Both CgB antibodies showed immunoreactivity in gastrin cells, intestinal (but not gastric) EC cells, pancreatic A and PP cells, pituitary TSH and gonadotrope cells and adrenal medullary cells. In addition, the B11 antibody stained thyroid C cells and the B13 antibody stained the Golgi area of pituitary GH cells. It is concluded that most CgB is stored in the same cells showing CgA, although some CgA-rich cells, like gastric EC and ECL cells, lacked B11 and B13 immunoreactivities and some CgA-poor cells, like human thyroid C cells, showed intense B11 immunostaining.

Chromogranin A (CgA), chromogranin B/secreto- granin I (CgB) and chromogranin C/secretogranin II (Sg II) belong to a family of anionic proteins known to be concentrated in secretory granules of most amine/peptide producing endocrine cells as well as in neuronal tissues (Fischer-Colbrie and Frisenschiøtz, 1985; Rosa et al., 1985; Benedum et al., 1986, 1987). Two peptides isolated from the pancreas, pancreastatin (Tatemoto et al., 1986) and β-granin (Hutton et al., 1987), have been shown to derive from CgA (Konecki et al., 1987); and two other peptides of pituitary origin, called GAWK and CCB, derive from CgB through processing at paired basic residues (Benedum et al., 1987; Benjannet et al., 1987). In addition, a 67KD anionic protein (and a related 35/32 KD dimer in reducing conditions) has been detected in secretory granules and Golgi cisternae/vesicles of many endocrine cells (Krisc et al., 1988).

All these secretory proteins and related peptides have proved to be useful markers of endocrine cells and tissues as well as of related endocrine growths (O'connor et al., 1983; Wilson and Lloyd, 1984; Hagn et al., 1986; Rindi et al., 1986; Solcia et al., 1986; Lloyd et al., 1988). However, the distribution of these proteins and peptides resulting from immunohistochemical studies has been shown to vary according to animal species investigated, antibodies/antiserum used, type of tissue fixation and processing employed (Grube et al., 1986; Hagn et al., 1986; Rindi et al., 1986; Lloyd et al., 1988).

*This investigation was supported in part by grants from the Italian Consiglio Nazional delle Ricerche (Special Projects “Biomedical Engineering” and “Oncology”).
Chromogranin Immunoreactivity of Human Endocrine Cells


Prof. Enrico Solcia
Department of Human Pathology
University of Pavia
Via Forlanini 16
27100 Pavia, Italy