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Occurrence of Both Growth Hormone- and Prolactin-Immunoreactive Material in the Cells of Human Somatotropic Pituitary Adenomas Containing Mammotropic Elements

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Summary. With the use of immunoperoxidase staining, both growth hormone- and prolactin-immunoreactivity were demonstrated in the cells of 6 pituitary adenomas removed because of frank or suspected acromegaly. By double immunostaining of individual sections or comparison of adjacent immunostained sections, partial to almost complete identity of the cell populations containing growth hormone and prolactin was found in 5 of the tumors.

Key words: Human pituitary adenomas – Immunocytochemistry – Growth hormone – Prolactin – Bihormonal cells

Recent publications (Lamberts et al. 1979; De Pablo et al. 1981) have indicated that hyperprolactinemia is present in 30–40% of patients with acromegaly caused by somatotropic pituitary adenomas. Furthermore, electron microscopic and immunocytochemical investigations have shown that acromegaly-producing pituitary adenomas which also contain neoplastic prolactin (PRL) cells (somato-mammotropic adenomas) are not uncommon (Guyda et al. 1973; Zimmerman et al. 1974; Corenblum et al. 1976; Halmi and Duello 1976; Duello and Halmi 1980). In addition, Peillon et al. (1980) have found that in organ culture even those somatotropic adenomas in which PRL cells were not detected by electron microscopic and immunocytochemical examination were able to synthesize and secrete PRL. According to Kovacs and his collaborators, there are 4 types of pituitary adenomas in which PRL may be or consistently is produced along with growth hormone (GH): a) somatotropic adenomas in which occasional groups of cells show staining for PRL in the Golgi region (Horvath and Kovacs 1980); b) mixed somato-mammotropic adenomas, which consist of a dual population of cells, GH and PRL cells, clearly separable by electron microscopic criteria (Kovacs et al. 1977; Horvath and Kovacs 1980); c) "acidophilic stem cell adenomas" derived from a hypothetical common progenitor of GH and PRL cells; the individual cells in these show ultrastructural characteristics of both GH and PRL cells (Horvath et al. 1977; Horvath et al. 1981); and d) "mammo-somatotroph cell adenomas", rare, well-differentiated tumors composed of cells that are said to contain both GH and PRL (Horvath et al. 1980).

The question of how consistently GH and PRL occur together in individual cells of somatotropic adenomas that also contain PRL has not been

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settled. For predominantly somatotropic adenomas with admixed mammotropic elements, it was postulated that cells showing immunostaining in the Golgi region are those that contain both hormones (Horvath and Kovacs 1980), but this was not documented. In tumors that qualify as mixed somatomammotropic adenomas, dissimilar distribution and shape of the two cell types was illustrated (Duello and Halmi 1980). It was stated that "GH and PRL seem to be in different cells" (Horvath and Kovacs 1980), a fact subsequently documented for several tumors (Martinez and Barthe 1982). However, the possibility that some cells may contain both hormones was not excluded, and this was indeed stated to be the case for 2 tumors, but the finding was not illustrated (Martinez and Barthe 1982). For "acidophilic stem cell adenomas" it was said, on the one hand, that "it [may be] difficult to establish whether the two hormones are present in the same cell". On the other hand, some tumors of this type were described as "containing areas in which every cell exhibits positivity for both hormones" (Horvath and Kovacs 1980). However, the accompanying illustrations show only, as the legend correctly states, that "immunoreactive PRL is detected in many of the cells containing immunoreactive GH". Finally, the description of "mammo-somatotroph adenomas" is thus far confined to an abstract, and the illustrations do not document the statement in the text that "both GH and PRL were present in the secretory granules of the same cell" (Horvath et al. 1980).

This paper described attempts to ascertain the occurrence of both GH and PRL in cells of pituitary tumors that were surgically removed because of proved or suspected acromegaly.

Materials and Methods

1. Materials

The tissues examined were biopsies of pituitary tumors removed because of acromegaly, at the University of Iowa (Cases 1–4, Table 1), because of acromegaly-gigantism, at Mount Sinai School of Medicine (Case 5, Table 1), or because of acromegaloid features, at Mount Sinai School of Medicine (Case 6, Table 1). The first 4 of these were fixed in Bouin's fluid and the remaining two in neutral buffered formalin. All were embedded in paraffin and sectioned at 4–5 μm. The salient clinical features are summarized in Table 1.

2. Methods

A. General

The methods used all involved the unlabeled antibody-peroxidase-antiperoxidase (PAP) complex technique of Sternberger et al. (1970). In all instances both GH- and PRL-immunoreactivity were demonstrable by immunostaining in the adenoma tissue, although in only 2 (Cases 1 and 4, Table 1) was plasma PRL elevated, in one mildly, in the other dramatically. In Case 6 plasma GH was normal on the day of the operation, and an alleged previous abnormally high value could not be located in the charts. The patient had definite acromegaloid features which persisted after the operation. Cases 1–4 were included in a previous paper (Duello and Halmi 1980), but a fifth case for which dissimilar morphology and distribution of GH and PRL cells was illustrated (Fig. 5 loco cit). is not part of the present series. For the demonstration of GH, rabbit anti-human (h) GH (NIAMDD Hormone Distribution Program, Beth-

Table 1. Clinical data on patients whose pituitary adenomas were studied. Upper limits of normal plasma hormone levels: GH, 10 ng/ml; PRL, 25 ng/ml

Case no.	Sex	Age, years	Diagnosis	Maximal preoperative plasma	
				GH	PRL
				ng/ml	
1	F	49	Acromegaly	235	36
2	F	21	Acromegaly	149	15
3	M	52	Acromegaly	205	22
4	M	59	Acromegaly	58	1,161
5	M	24	Acromegaly = gigantism	150	<25
6	F	49	Acromegaly?	6?	7

esda, MD, USA) was used as the primary antibody at a dilution of 1:1000 in Tris-buffered saline with 1% normal sheep serum (TBSS), pH 7.6, for 24 h at 4° C. No staining was seen when the antiserum was absorbed for 48 h before its application to the slides, and when it was layered on them, with excess hGH (1 μg/ml) (specificity control). For the demonstration of PRL and the documentation of the specificity of the staining, the same protocol was followed, but with rabbit anti-hPRL and hPRL (both from the NIAMDD) substituted in the same concentrations for the anti-hGH and hGH, respectively. The secondary antibody was applied after the sections were flooded with TBSS for 10 min at room temperature, and consisted of sheep anti-rabbit IgG (Antibodies, Inc., Davis, CA, USA) (1:50 in TBSS, exposure time 10 min at room temperature). A rinse with Tris-buffered saline (TBS) was followed by a 10-min application, also at room temperature, of a 1:50 dilution of rabbit PAP complex (Miles Laboratories, Elkhart, IN, USA) in TBS. Then the sections were transferred to phosphate-buffered saline (PBS), pH 7.6. Peroxidase activity was revealed with 3,3′-diaminobenzidine (DAB) dissolved in PBS (30 mg/100 ml), with 0.17 ml 30% H_2O_2 per 100 ml added. When double immunostaining was used, the other chromogen was 4-chloro-1-naphthol (CN) (50 mg/100 ml PBS with 0.03% H_2O_2) in addition to DAB. Development of staining was followed under a low-power microscope.

B. Comparison of Adjacent Immunostained Sections

For this, the neighboring sections have to contain landmark structures (such as blood vessels) that permit accurate lining up of corresponding fields.

Method I. The preferred method was an attempt to identify individual adenoma cells in adjacent sections stained for GH and PRL, respectively. This technique is most successful when the immunostained cells are relatively few, regularly shaped, and in good contrast with a minimally stained background. An acetate sheet was superimposed on a 20 × 25 cm print of the section stained for PRL and all the immunoreactive cells were outlined. By laying the transparent sheet thus marked over a 20 × 25 cm print of the section stained for GH, after lining up on the basis of the landmarks common to the 2 prints, it was possible to identify all PRL-positive cells that were also reactive for GH (Fig. 1).

Method II. If the immunostained cells were irregular, crowded, and not clearly oulined everywhere, a less straight-forward method had to be resorted to. This consisted of placing transparent graph paper with a 75 × 100 mm grid over the print of the section stained for PRL, and marking all of the 7500 squares (1 mm^2 each) of the grid that overlay PRL-positive cells. After careful alignment of the guiding landmarks with those in the print of the adjacent section stained for GH, this process was repeated and the squares corresponding to GH-positive

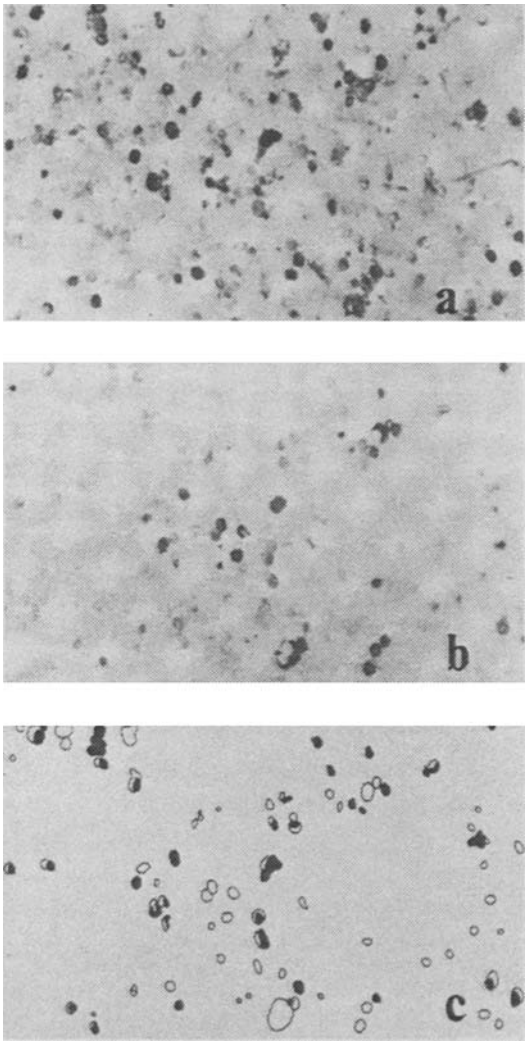

Fig. 1. a Adenoma tissue from Case 2, stained with anti-GH. **b** Corresponding field from an adjacent section stained with anti-PRL. **c** Tracing showing pure PRL cells in panel **b** (empty symbols) and those which also stain for GH (solid symbols). ×150

material were marked in a different color. By subsequently identifying the squares marked for both GH and PRL staining, a measure of their mutual overlap in the tumor could be obtained (Fig. 2). This method, in addition to being time-consuming, has the disadvantage of using a relatively coarse grid, so the extent of GH- and PRL-immunostaining and their overlap are systematically overestimated. It does, however, permit statistical analysis. Each of the 1-mm wide rows of the grid is treated as a unit. If the overlap between GH- and PRL-staining were random, it should be = [percentage of squares positive for GH × percentage of squares positive for PRL] (e.g., 40% × 40% = 16%). If the actual overlap for each row is compared with this value, the significance of deviation from randomness can be assessed with the paired *t* test. A significant excess of found over random overlap indicates that GH and PRL coexist in identical cells. A control for this calculation can be obtained by lining up the grids so the landmarks do not match, in which case the overlap between GH-positive and PRL-positive squares should be no better than random even if the 2 hormones do occur in the same cells.

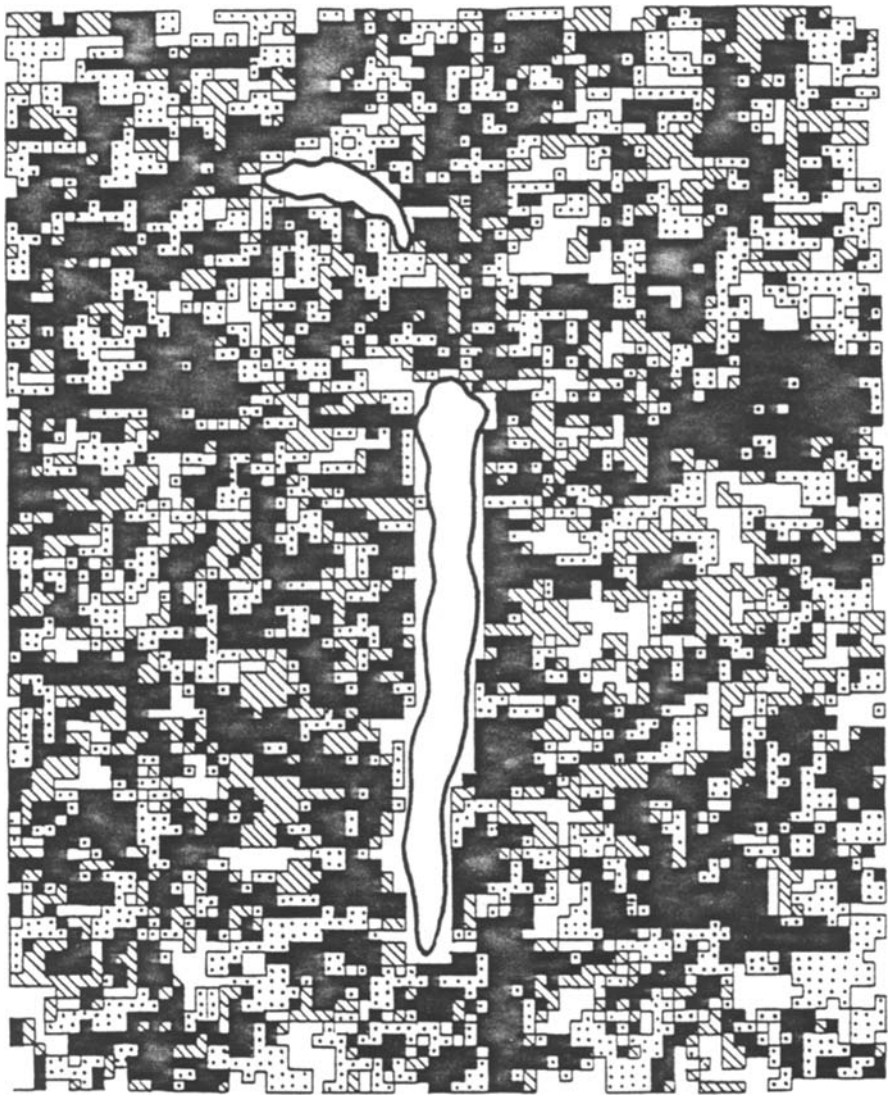

Fig. 2. Grid illustrating overlap between PRL- and GH-immunoreactivity in tissue from Case 3. Landmark vessels comon to adjacent sections are outlined. *Dotted areas* indicate reactivity for PRL only, *hatchmarked areas* reactivity for GH only, and black areas reactivity for both

C. Double immunostaining

Method 1: With Removal of the First Antibody. For this method, the chromogen used with the first antibody is CN. The blue color thus obtained is alcohol-soluble, and the image has to be photographed promptly after the coverslip is mounted with glycerin. The color is then extracted by running the slide through an ethanol series to distilled water. The first antibody is subsequently removed with 0.08% $KMnO_4$ in 0.03% H_2SO_4 (1 min), after which the section is bleached in 5% $Na_2S_2O_5$ for 5 min, thoroughly washed in distilled water, and transferred to TBSS. Then the second antibody is applied, and its sites of interaction with the appropriate antigen are demonstrated with DAB. The brown reaction product of DAB

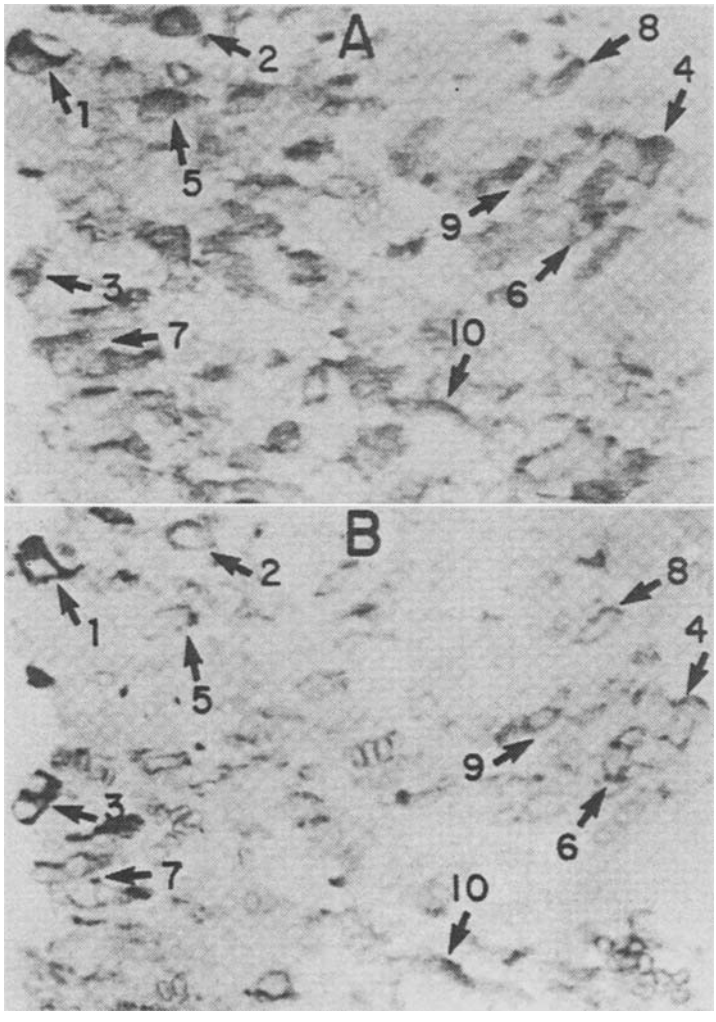

Fig. 3. A Adenoma tissue from Case 3 stained with the anti-PRL-CN sequence. **B** The identical field stained, after removal of the first antibody, with the anti-GH-DAB sequence. Ten identical cells stained with both antibodies are indicated by the *numbered arrows*. × 350

peroxidation being resistant to alcohol, the slide can now be coverslipped in the conventional manner, and an image of the same field as that photographed after CN staining obtained (Tramu et al. 1978; Nilaver et al. 1979; Teitelman et al. 1981). To rule out non-specific staining, each of the antigen-antibody interactions must be successfully blocked in control sections by an excess of the appropriate antigen. This method gives unequivocal results when it works well, since it permits cell-by-cell comparisons in black- and-white prints (Figs. 3 and 6). However, strong antigen-antibody linkages may be hard or impossible to disrupt with the acidified $KMnO_4$ (Sternberger and Joseph 1979). In our experience the main problem was that in some instances this harsh treatment evidently denatured the antigen that was to be demonstrated with the second antibody.

Method II: Without Removal of the First Antibody. This form of double immunostaining was performed by the technique of Sternberger and Joseph (1979). The sites of interaction

of the first antibody with its antigen are localized with DAB, after which the antibody is not removed. The second antibody, followed by CN, then yields blue staining of cells containing only the antigen against which this antibody is directed, and mixed staining in various shades ranging from blue to brown in cells that contain both antigens. The main problem with this elegant method is that distinction between cells that stain brown with DAB and cells showing faint admixture of blue due is CN is highly subjective. In fact, we have found it advisable to stain sections of the same tissue with anti-PRL and CN or anti-GH and CN as the second step, since the distinction of monohormonal cells from bihormonal cells was much easier when the former stained blue with CN rather than brown with DAB (Fig. 4a–d).

Results

Case 1. In this tissue GH cells were greatly in excess of PRL cells. Both antibodies stained the cytoplasm of the tumor cells diffusely. Double immunostaining by Method C. II. yielded no clear evidence of bihormonal cells. Figure 4a shows some PRL cells stained blue with the anti-PRL-CN sequence, and the predominant GH cells stained brown with the anti-GH-DAB sequence. The same duality of cells was seen when the anti-GH was followed by CN and the anti-PRL by DAB. Of course, it cannot be ruled out that bihormonal cells did exist in portions of the tumor not sampled.

Case 2. In this tumor too, GH cells (Fig. 1a) outnumbered PRL cells (Fig. 1b), but even the GH cells were relatively sparse. Further, all cells had distinct boundaries, fairly regular shapes, and good contrast with the background, which permitted analysis by Method B. I. on adjacent sections. This revealed that GH-immunoreactivity was also present in about one third of the cells positive for PRL (Fig. 1c). Double immunostaining by Method C. II. confirmed the existence of pure PRL cells in the tumor, and the higher number of cells positive for GH or GH and PRL (Fig. 4b). Attempts to use double immunostaining with removal of the first antibody (Method C. I.) failed because of destruction of immunogenicity of the second antigen.

Case 3. Owing to the lack of clear-cut landmarks, comparison of adjacent sections could not be performed, nor was Method C. I. successful, for the same reason as in Case 2. Double immunostaining by Method C. II. (Fig. 4c) showed that the vast majority of tumor cells stained in shades intermediate between blue and brown, whether anti-GH or anti-PRL was used as the first antibody (followed by staining with CN). This indicated therefore that the adenoma was composed predominantly of bihormonal cells.

Case 4. The adenoma in this instance was very rich in irregularly shaped cells, whether it was immunostained for PRL or GH. GH-staining was more intense. Analysis by Method B. II. showed 66% of the area under the grid to be positive for PRL and 57% for GH. Overlap was 41% of the total area (Fig. 2), which was significantly greater than the random value ($t = 11.8$, $p < 0.001$). Double immunostaining by Method C. I. (Fig. 3) showed unequivocal identity of many GH- and PRL-positive cells, but nu-

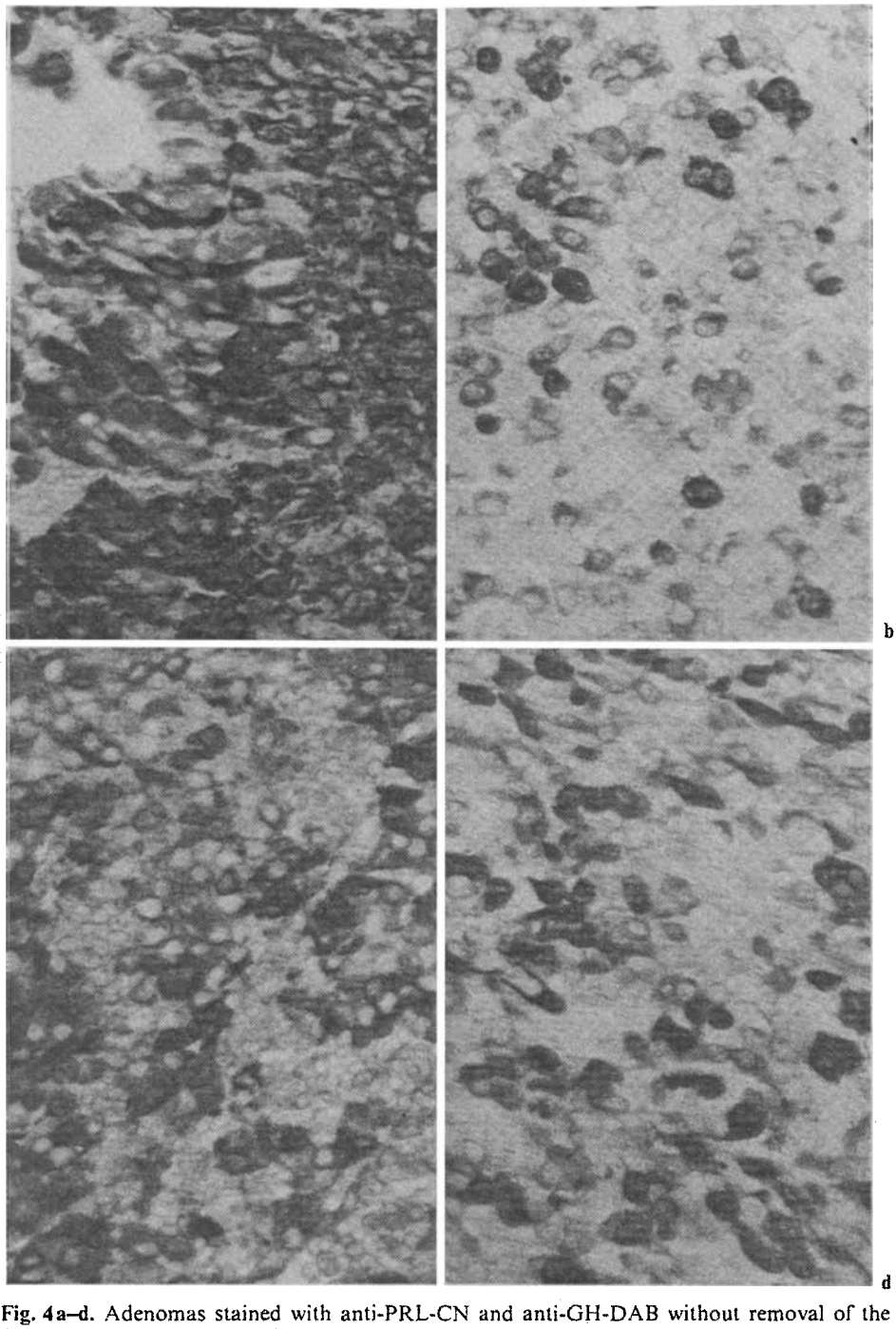

Fig. 4a–d. Adenomas stained with anti-PRL-CN and anti-GH-DAB without removal of the first antibody. **a** Case 1. A few cells are stained blue with the first sequence only. Most are positive (brown) for the second sequence. **b** Case 2. Cells stained blue for PRL are outnumbered by cells stained brown for GH or in intermediate shades for a mixture of the 2 hormones. **c** Case 3. All cells strain with both sequences, in shades intermediate between blue and borwn, indicating the presence of both PRL- and GH-immunoreactivity in them. **d** Case 4. Two cells stain blue for PRL only, the rest brown for GH, or in intermediate shades for GH plus PRL. Original × 313

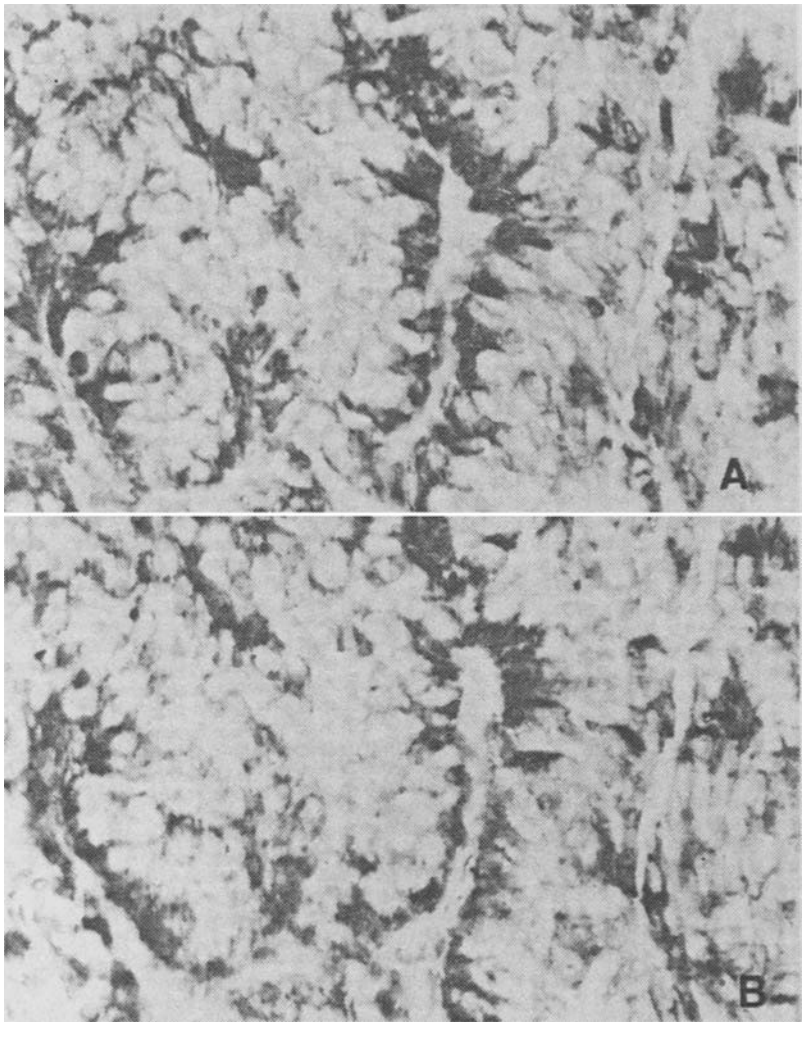

Fig. 5. Corresponding fields in adjacent sections from the tumor in Case 5 stained for GH (panel **A**) and PRL (panel **B**). Closely similar distribution of immunoreactivity is evident. ×450

merous cells that stained for PRL did not stain for GH. Double immunostaining by Method C. II. documented occasional cells positive for PRL only (Fig. 4d), but none that contained GH alone when the anti-GH-CN sequence was applied first. The tumor was thus revealed as being mostly bihormonal, but with some monohormonal cells containing PRL only.

Case 5. The tumor in this case was composed of cells that were elongated and showed distinct polarity. Immunoreactivity for both GH and PRL was generally confined to the ends of the cells that abutted on the connective tissue septa. In corresponding areas of adjacent sections stained for GH and PRL, respectively, very similar patterns of immunoreactivity could be

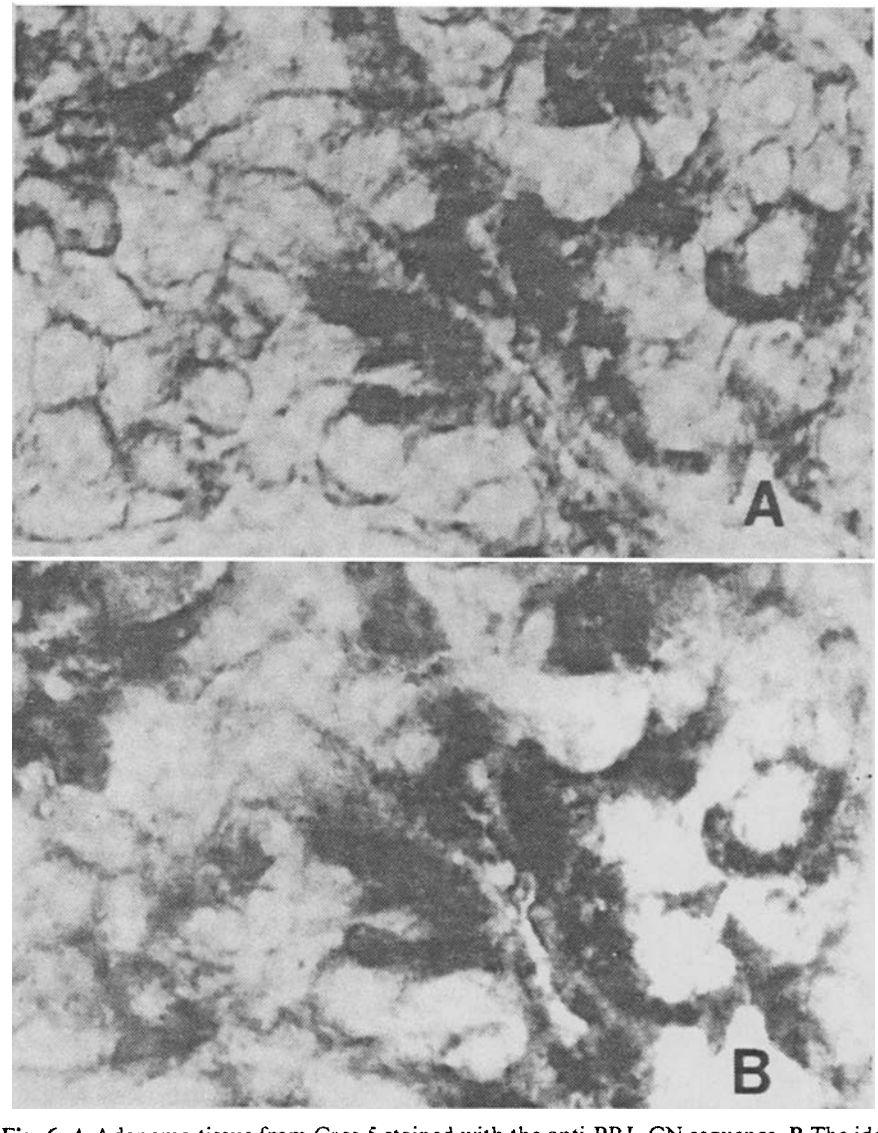

Fig. 6. A Adenoma tissue from Case 5 stained with the anti-PRL-CN sequence. B The identical field stained, after removal of the first antibody, with the anti-GH-DAB sequence. Almost all cells in the field stain with both antibodies. ×900

discerned (Fig. 5). This was confirmed by tracing (Method B.I.). Furthermore, double immunostaining by Method C. II. showed that the vast majority of tumor cells stained in shades between brown and blue, i.e., for both GH and PRL. Double immunostaining by Method C.I. was also successful in this instance, and documented widespread identity of adenoma cells that were positive for GH and PRL (Fig. 6). For this adenoma, very strong evidence was thus obtained that, like the one in Case 3, it was composed largely of bihormonal cells.

Case 6. The tissue suffered from suboptimal fixation, and was composed of cells highly irregular as to shape and intensity of immunostaining. The only method applicable to it was Method. B.II. Areas positive for GH and PRL were about the same (49% vs. 50%), and the observed overlap (30% of the grid area) exceeded random overlap significantly ($t=6.5$, $p<0.001$). When the grid was aligned out of phase with respect to the landmark vessel common to the GH- and PRL-stained adjacent sections, overlap was less than random by a value of borderline significance ($t=1.6$, $p=0.06$). On the basis of this circumstantial evidence, it was concluded that in at least some of the cells of this tumor GH and PRL must have occurred together.

Discussion

The simultaneous occurrence of GH and PRL in individual adenoma cells of the 6 tumors constituting this series ranged from not demonstrable (Case 1) through probable (Case 6) and unequivocal (Cases 2 and 4) to almost universal (Cases 3 and 5). The only convincing previous illustration of the presence of both these hormones in the same cells was for an "acidophilic stem cell adenoma" (Horvath and Kovacs 1980). In the two mixed somato-mammotropic adenomas in which both GH and PRL were said to be stored in the same cells, the supporting figures lack landmarks that would allow conclusive identification of matching cells in the adjacent sections (Girod et al. 1980; Kameya et al. 1980). It is unlikely that the 4 cases in this series in which the presence of both GH and PRL in the same cells was clear-cut (Cases 2–5) were "acidophilic stem cell adenomas", since plasma GH levels in association with such tumors are generally in the normal range (Horvath et al. 1981), whereas in the present series plasma GH was markedly elevated in all instances but Case 6. It is also highly dubious that all our cases with GH and PRL in the same tumor cells were "mammo-somatotroph cell adenomas", since such tumors are said to be rare. From the brief description available at this time, it is not clear, however, whether they can even be identified without the aid of electron microscopy (Horvath et al. 1980), so the possibility that our tumors with bihormonal cells were, against statistical odds, all "mammo-somatotroph adenomas" cannot be ruled out. It is nevertheless more likely that GH- and PRL-immunoreactive material can be found together in cells of more common varieties of tumors associated with frank acromegaly, if an effort is made to identify such bihormonal cells.

As is usually the case when immunocytochemistry is not complemented by biochemical and bioassay evaluation of the tissue, one cannot be sure that the PRL-immunoreactive material demonstrated was authentic, secreted and biologically active PRL. In only one instance (Case 4) was marked hyperprolactinemia shown by radioimmunoassay, and the clinical records contained no evidence for any biological effect of the great excess of circulating PRL in the 59-year-old male patient.

The concept that GH and PRL cells, although in general they are morphologically quite distinct (Halmi et al. 1975), may arise from a common

progenitor (Horvath et al. 1977) is quite attractive. The 2 hormones share extensive amino acid sequences (Shome and Parlow 1977), and a common phylogenetic ancestry has been postulated for them (Niall 1976). It therefore appears reasonable that GH cells might in the course of neoplastic transformation dedifferentiate to a stage where they produce PRL or PRL-like material together with GH, even if they would normally be committed to the production of GH only. Further, it may be that differentiated GH-PRL cells also occur in the normal human hypophysis. This was postulated by Zimmerman et al. (1974), who found that in some human pituitaries the count of immunoreactive GH and PRL cells added up to over 100% of all adenohypophysial cells. Generally, however, GH cells constitute some 40% and PRL cells about 20% of the cell population of the human anterior lobe, so the circumstantial evidence for CH-PRL cells offered by Zimmerman et al. must be viewed with some reservation. Such cells have since been seen by others too, although not yet described in detail (Horvath et al. 1980; Landolt 1981, unpublished observations). As soon as there is cogent documentation for the existence of differentiated GH-PRL cells, they will have to be considered logical precursors of at least some of the adenomas in which individual tumor cells contain both hormones.

The final question is whether GH and PRL, when they occur concomitantly in adenoma cells, are in separate sets of granules. On suitably preserved material, this can be best answered with the use of double-labeling immunoelectron microscopic methods utilizing colloidal gold particles of 2 sizes, such as those developed by Roth et al. (1978), Larsson (1979), and Geuze et al. (1981).

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