Immunohistochemical and stereological study of neuroendocrine cells in human antrum during the perinatal period

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ABSTRACT **A correlative immunohistochemical and stereological study of neuroendocrine cells (NEC) was carried out in the antrum of twenty human fetuses with gestational ages from 18 to 42 weeks and of two specimens postnatally. Neuron-specific enolase (NSE) as a common marker of neurons and NEC, as well as gastrin (G-) and somatostatin (D-) immunoreactive cells served for evaluation of volume density, which proved to be the most convenient method for quantitative analysis of NEC. It was observed that a considerable frequency of NEC appeared at 23-24 weeks of gestation (8% of NSE- and 6% of G- cells) and coincided with the adult pattern of intramural innervation. After a repeated increase of NEC in the 26-week-old fetus, the frequency of NEC remained persistant during the perinatal period (10-12% of NSE- and 7-8% of G- cells). An exception was a specimen with a prolonged pregnancy (42 weeks) in which the percentage of NSE- (17%) and G- (10%) cells was almost the same as at 6 weeks postnatally. The maximal quantitative difference of NEC was noted between 6- and 8-week specimens postnatally, e.g. 9% to 22% of G- cells, respectively. Observations obtained by NSE and S-100 protein were also demonstrated in lymphoid cells of gut associated and mesenteric lymphoid tissue.**

KEY WORDS: *human antrum, perinatal period, neuroendocrine cells, stereology, immunohistochemistry*

Introduction

The multistep process of morphogenesis and differentiation of the gastrointestinal tract proceeds in a specific and spatial pattern, although the existence of a single versus a dual gradient hypothesis of enteric neuronal development is still discussed (Okamoto and Ueda, 1967; Andrew, 1971; Tam and Lister, 1986). These processes are accompanied by epithelial-mesenchymal and cell interactions and by trophic actions of gastrointestinal peptides, in particular gastrin (Johnson, 1976, 1987). In addition, examples of long-term trophic roles of neuropeptides have also been established (Bloom and Polak, 1978; Burnstock, 1982). Apparently, the morphological and functional maturation of the vertebrate gut is under multihormonal control in which the entero-endocrine system plays a significant role (Bloom and Polak, 1978). Furthermore, differentiation of enterocytes from stem cells to highly differentiated epithelial cells occurs during ontogenesis but also with adolescence by continuous cell renewal (Dauça *et al.*, 1990).

Despite numerous studies on the diffuse neuroendocrine system in vertebrates (Buchan and Polak, 1980a; Solcia *et al.*, 1981; Pearse, 1986) little is known about the ontogeny of this system in the human gut. The developmental stage at which the peptides of the human gut neuroendocrine system are first recognizable has been better established in the intestine than in the antrum (Buchan and Polak, 1980b; Bryant *et al.*, 1982; Facer *et al.*, 1985; Larsson *et al.*, 1987).

Since peptide immunocytochemistry does not allow demonstration of neuroendocrine cells at one time, the immunodetection of neuron-specific enolase (NSE) as a common marker of these cells permits an integrated study of both enteric neurons and neuroendocrine cells (Bishop *et al.*, 1982).

In view of the fact that quantitative data on neuroendocrine cells in fetal gut segments are scarce or even nonexistent, a correlative immunohistochemical and morphometric study was indicated to investigate the development of the neuroendocrine system during the late fetal period.

For this purpose the frequency of neuroendocrine cells displaying NSE immunoreactivity as well as gastrin and somatostatin producing cells in human antrum was investigated during the

Abbreviations used in this paper: AEC, 3-amino-9-ethylcarbazde; DAB, 3,3'-diaminobenzidine; NSE, neuron-specific enolase; NEC, neuroendocrine cells; PAP, peroxidase-antiperoxidase; V_V, volume density.

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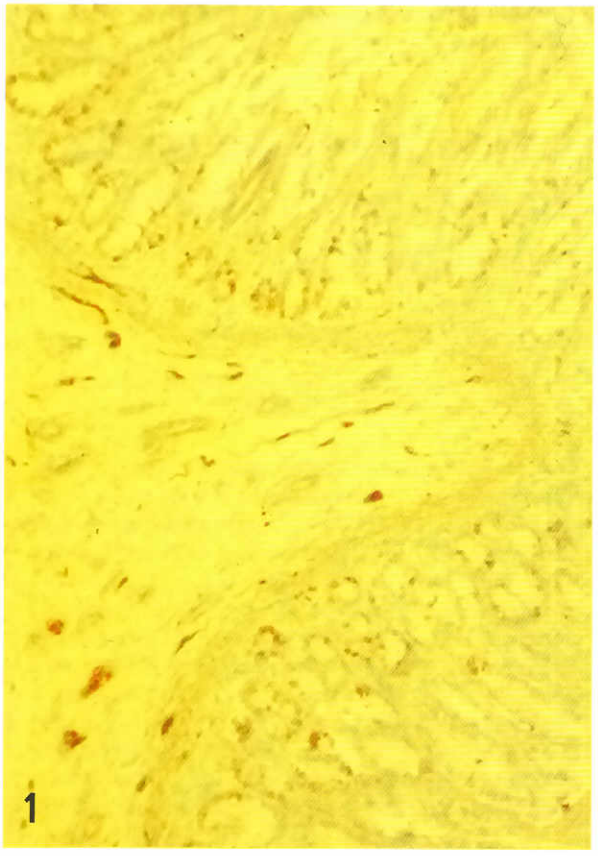
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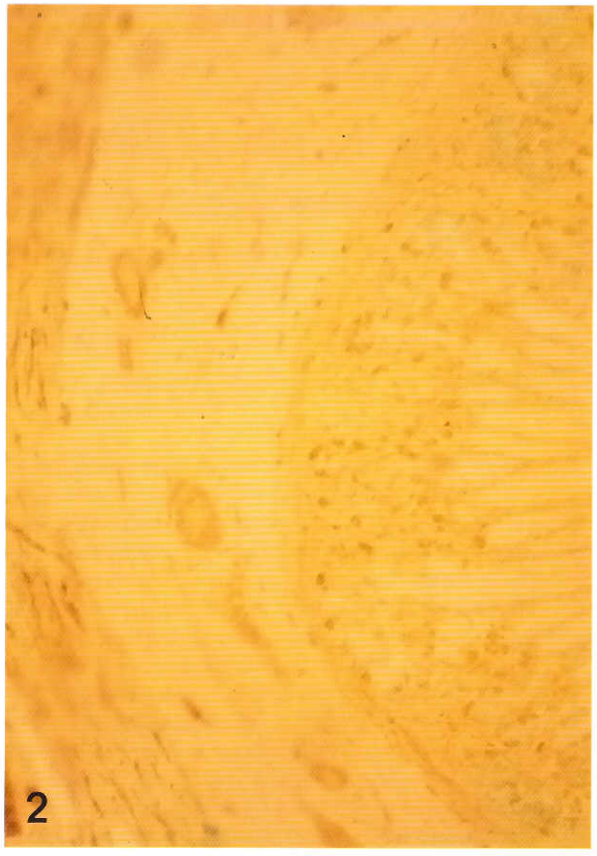
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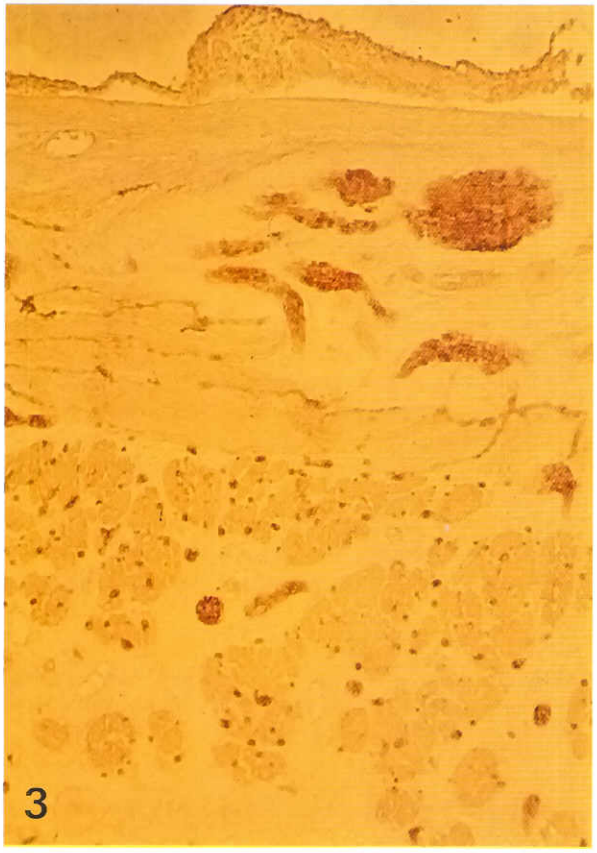
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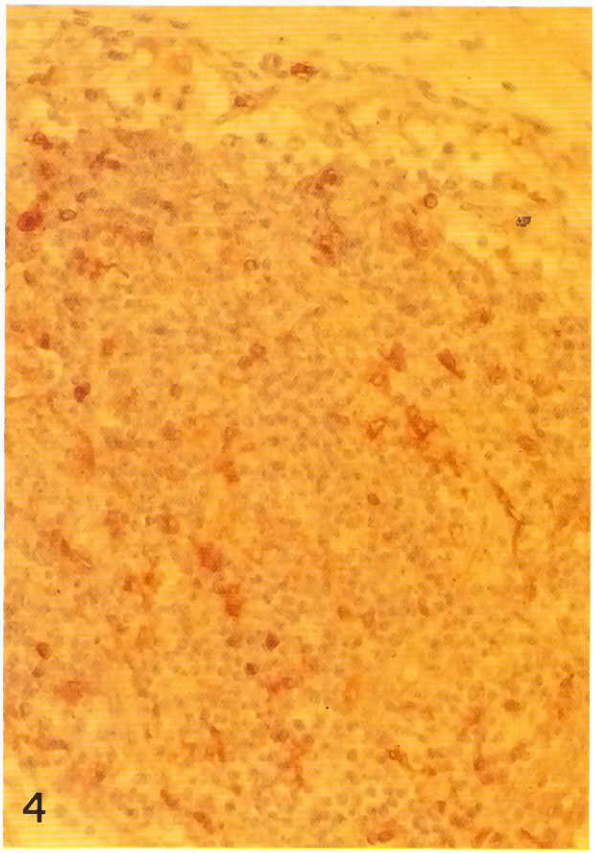
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midtrimester and perinatal period. Tissue specimens from two infants were also included in this study. The immunohistochemical method for S-100 protein served as a complementary marker for enteric nerves.

Results

Neuron-specific enolase (NSE) immunoreactivity, which served as a common marker of enteric nerves and neuroendocrine cells, revealed at 18 and 21 weeks of gestation only single scattered cells that did not exceed 30 cells per square millimeter. At 23-24 weeks, NSE-immunoreactive neuroendocrine cells with various grades of granular density reached 8% of total epithelial cells with a further increase to 10-12%, which persisted during the perinatal period (Figs. 1 and 2). A maximum of 17% of neuroendocrine cells was found in antral mucosa in specimen with prolonged pregnancy (42 weeks). In a postnatal 6-week-old specimen, the frequency of NSE-cells was nearly the same (16%) and at 8 weeks 25% of NSE-cells was found in the antral mucosa.

Examination of serial sections of the antrum and adjacent proximal duodenum showed 18- and 21-week-old fetuses in which both myenteric and submucous plexuses were better developed in the duodenum, especially in the submucous plexus. After 23-24 weeks of gestation, when the adult pattern of intramural nerves was established in the antrum (Fig. 3), no major differences with respect to time of appearance of enteric innervation were observed between these two gut segments.

Immunohistochemical demonstration of the S-100 protein, which served in this study as an additional marker of enteric nerves, confirmed the observations obtained with NSE. S-100 protein immunoreactive lymphoid cells and possibly dendritic reticular cells were found in gut associated and mesenteric lymphoid tissue during the investigation period (Fig. 4).

In the fetal antrum, gastrin (G-) cells were small, piriform, and localized first in the lower third and later preferentially in the middle third of the antral glands (Figs. 5-7). At 18 and 21 weeks they were similar to NSE-reactive cells, scarce per unit area and not suitable for stereological analysis. At 23-24 weeks, as shown in the graph, the frequency of G-cells reached 6% of total epithelial cells and persisted at the level of 7%-8% G-cells during the perinatal period with the exception of one specimen with prolonged pregnancy of 42 weeks (10% G-cells). An extreme difference in the frequency of G-cells was found postnatally between 6-week and 8-week-old specimens with 9% and 22% of G-cells, respectively.

Immunostaining experiments for gastrin by changing sodium chloride concentrations in PBS (0.15 to 0.5 M) as well as changing the molarity of phosphate buffer (0.01 M to 0.15 M) at pH 6.5 and pH 7.2 did not show the effect of nonspecific immunostaining due to the ionic interactions of immunoglobulins, described by Grube in rats (1980).

Immunoreactive cells to somatostatin (D-cells), mainly localized in the basal part of antral glands (Fig. 8), displayed characteristic morphological features including cytoplasmatic elongations. Somatostatine-containing nerves, abundant in the adult gut, were not observed in the investigated material. Inspite of material scarcity for somatostatin immunoreactions, a maximum of 8% of D-cells was registered in the specimen with prolonged pregnancy, while in the perinatal period it ranged from 4%-6% of D-cells.

Volume density (Vv) of NSE-, gastrin and somatostatin immunoreactive cells is presented in Table 1 and Fig. 9.

Discussion

Immunocytochemical identification of neuroendocrine cells in the gastrointestinal tract, producing more than 25 peptides (Polak *et al.*, 1981) reached culmination in the past decade. However, the ontogeny of this «largest endocrine gland» is still obscure, due to the scarcity of information on the distribution pattern and sequential frequency of specific neuroendocrine cells in gut segments.

According to Buchan and Polak (1980b) at 8 weeks endocrine cells containing gastrin, somatostatin, motilin and GIP are already present; at 11 weeks cells containing neurotensin and enteroglucagon were observed together with VIP-containing nerve fibers; and at 13 weeks CCK and secretin-producing cells were demonstrated. Bryant *et al.* (1982), studying endocrine cells and nerves in the gut of human fetuses from 8 weeks to term, found a well-developed network of nerves at the earliest stage (8 weeks). They also observed that, at 11 weeks, the distribution of endocrine cells was similar to that of adults. On the other hand, Larsson *et al.* (1987), studying eight regulatory peptides in the intestine of human fetuses with gestational ages between 8 weeks and 40 weeks, found that no peptide-containing neurones could be detected before the 14th week of gestation, after which a rapid development of endocrine cells and nerves was seen. Apparently, the chronology of events during the ontogeny of neuroendocrine system in the gut is not consistent. Moreover, scarce and fragmentary data exist on the development of the neuroendocrine system during the late fetal period, particularly in the pyloric antrum. Tam and Lister (1986) studying NSE-immunoreactivity by an indirect immunofluorescent method in human gut during 9-21 weeks of gestation did not mention the existence of any endocrine cells.

In our opinion, the interpretation of immunocytochemically determined neuroendocrine cells in the gut during ontogeny may gain much in value when quantitative analyses are used as well.

To our knowledge, there are few studies which have employed an acceptable quantitative technique to assess neuroendocrine cells of the gut during ontogeny. Creutzfeldt *et al.* (1976) on animals and Singh (1965) and Bryant *et al.* (1982) on human fetal material estimated the number of cells per unit area (mm^2). The advantages and disadvantages of these morphometric methods have been discussed in more detail elsewhere (Hobbs and Polak, 1978; McIntire and Piris, 1981; Fujimura *et al.*, 1987).

According to our experience the stereological methods have proved to be more accurate for quantification of neuroendocrine

Figs. 1 and 2. NSE-immunoreactive endocrine cells situated in the lower part of the antral mucosa at 38 week of gestation. *PAP method with AEC. x60 (Fig. 1) and DAB as chromogen. x120 (Fig. 2).*

Fig. 3. NSE-immunoreactive intramural innervation showing adult pattern at 24 week old fetus. *PAP method with DAB as chromogen. x60.*

Fig. 4. S-100 protein immunoreactive lymphoid cells (reddish stained) in gut associated lymphoid tissue. *PAP method with AEC as chromogen. x120.*


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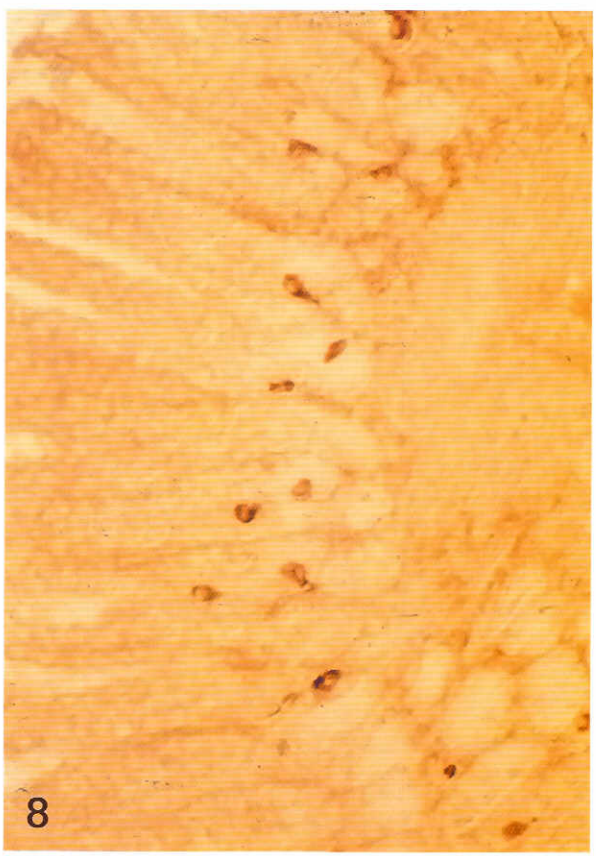
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cells in gut segments (Silek-Saran *et al.*, 1979; Lackovic *et al.*, 1985; Rode *et al.*, 1989). For this purpose volume density (V_v) has an advantage over numerical density (Lackovic *et al.*, 1985) because the frequency is less dependent on the precise thickness of specimens and can be expressed in the percentage (V_v%) of endocrine cells. As stated by Weibel (1981) no problems arise when estimating volume densities, because this measurement is unbiased.

In general, quantitative estimation of neuroendocrine cells is hazardous because of the irregular distribution of these cells in the gut. One should remember that during fetal life considerable redistribution of enteroendocrine cells with sequential appearance of particular molecular forms of peptides, e.g. gastrin, occurs (Larsson, 1977). Sampling error is probably the major factor accounting for some of the conflicting results obtained by quantitative studies.

In this stereological study we have used gastrin and partly somatostatin containing cells as principal functional cells of the antrum and NSE-immunoreactivity as a common marker of neuroendocrine cells. At 18-21 weeks of gestation NSE- and G-cells were scarcely distributed and sampling did not allow reliable stereological analysis. Their frequency was about 30 cells per square millimeter. As shown in Fig. 9, the volume density of NSE and gastrin immunoreactive cells reached an appreciable 6%-7% cells at 23-24 weeks of gestation, when the enteric neuronal pattern is fully established. The tendency of a moderate increase from 26 weeks to 30 weeks and then a stabilization of volume density of gastrin and NSE-immunoreactive cells is quite obvious with an exception in the fetus with prolonged pregnancy. It should be noted that a similar tendency was observed during stereological analysis of argyrophil cells demonstrated by the method of Grimelius (Lackovic, unpublished data).

The observations presented here are in accordance with the opinion of Bryant *et al.* (1982) that substantial development of the gut neuroendocrine system, which occurred by 25 weeks in the intestine, represents the earliest time at which the fetus may first be able to support itself by oral feeding if born prematurely. Our findings in the antrum show that the adult pattern may be established even earlier, at 23-24 weeks, which is already a matter of gradient hypothesis.

Concerning the multistep process of morphogenesis and differentiation of the gut, the spatial and chronological appearance of enteric ganglia is still hypothetic. Okamoto and Ueda (1967) demonstrated a craniocaudal gradient of migration of neuroblasts in human embryonic gut, and concluded that agangliosis in Hirschprung's disease was a developmental anomaly, expressed in arrest of this neuroblast migration. This hypothesis was also experimentally supported by Webster (1973) in mice. In contrast, a number of workers found in their animal experiments a dual origin and gradient of neuronal development of enteric ganglia, proceeding from both ends to the middle of the gut (Andrew, 1971). This hypothesis was later also supported by experimental work in the chicken (Gershon *et al.*, 1980). Tam and Lister (1986), who studied, as mentioned above, NSE in fetal gut, found that the development of the enteric nervous system was shown to be the most advanced in pylorus, less so in the colon, and least so in the ileum. The authors concluded that these findings support the hypothesis of a dual gradient of neuronal development in human fetuses.

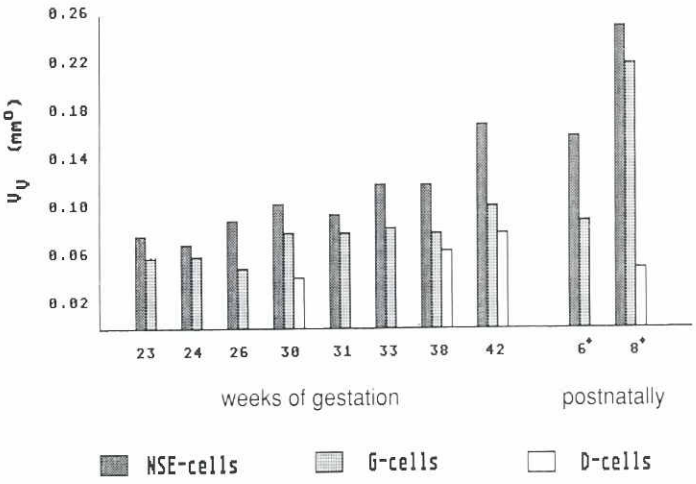

Fig. 9. Volume density (Vv) of NSE-, G- and D-cells in fetal antrum from 23-42 weeks of gestation and in postnatal period of 6* and 8* weeks.

According to observations presented here the myenteric and especially submucous nerve plexuses are better developed in the proximal duodenum than in the antrum until 21 weeks of gestation. With a considerable increase of neuroendocrine cell number at 23-24 weeks these differences disappear. The mentioned findings are apparently not in agreement with the craniocaudal gradient concept.

NSE as a common marker of the diffuse neuroendocrine system was first considered to be strictly localized in neurons, indicating that the gene coding for the gamma subunit is only expressed in neuronal cells (Marangos *et al.*, 1978). Schmechel *et al.* (1978) showed, however, that neuroendocrine cells belonging to both central and peripheral divisions, including the gut, also contained NSE. However, according to Pearse (1986), there is one significant difference between neurons and neuroendocrine cells in that NSE appears in the former only when they cease to divide and begin to make synaptic contacts. In various neuroendocrine cells of the mammalian fetus, however, NSE appears long before those cells cease to divide. In neurons NSE is thus a marker for the late transformation of a cell committed for neural function, while in neuroendocrine cells it should be interpreted as an indicator of the onset of neuroendocrine determination. If this is correct, the quantitative data presented here concerning the corresponding frequency of NSE and peptide producing cells e.g. G-cells, are in complete agreement with the above interpretation.

Although the role of NSE and S-100 protein in both neurons and neuroendocrine cells has not been clarified, apparently a complete assembly of intramural innervation and neuroendocrine cells simi-

Figs. 5-7. Gastrin cells in antral mucosa at 23 weeks (Fig. 5) and 33 weeks of gestation (Fig. 6) and at 8 weeks postnatally (Fig. 7). *PAP method with DAB as chromogen. x120.*

Fig. 8. Somatostatin (D-) cells in the basal part of the antral glands at 30 weeks of gestation. *PAP method with DAB as chromogen. x240.*

TABLE 1

VOLUME DENSITY ($V_V$mm°) OF NSE-, GASTRIN (G-) AND SOMATOSTATIN (D-) IMMUNOREACTIVE CELLS (MEAN ± SE) IN FETAL ANTRUM FROM 23-42 WEEKS OF GESTATION AND IN POSTNATAL PERIOD OF 6* AND 8* WEEKS

Age (weeks)	Field number «n»	V_V of NSE cells (mm°)	V_V of G-cells (mm°)	V_V of D-cells (mm°)
23	140	0.075 ± 0.008	0.058 ± 0.03	-
24	140	0.068 ± 0.006	0.060 ± 0.004	-
26	50	0.088 ± 0.012	0.049 ± 0.005	-
30	60	0.108 ± 0.007	0.077 ± 0.008	0.043 ± 0.005
31	140	0.095 ± 0.006	0.078 ± 0.007	-
33	50	0.116 ± 0.004	0.084 ± 0.004	-
38	50	0.121 ± 0.005	0.079 ± 0.007	0.064 ± 0.003
42	50	0.170 ± 0.013	0.103 ± 0.009	0.080 ± 0.003
6*	50	0.160 ± 0.008	0.089 ± 0.008	-
8*	50	0.254 ± 0.011	0.221 ± 0.011	0.050 ± 0.003

*postnatally

lar to that in postnatal life occurred in the antrum at 23-24 weeks of gestation. The presence of S-100 protein as a «second marker» of the autonomic nerve differentiation supports this finding. S-100 protein, which was initially identified in the glial cells of the central nervous system and later also in peripheral nerve Schwann's cells (Kahn *et al.*, 1983), was demonstrated in cells of lymphoid tissue adjacent to fetal gastro-duodeno-pancreatic region (Rode *et al.*, 1989).

Protein S-100 immunoreactivity was recently demonstrated in subpopulations of lymphocytes (Takahashi *et al.*, 1985) and in interdigitating reticulum cells of lymph nodes (Takahashi *et al.*, 1981). Furthermore, Ferrari *et al.* (1988) found that one half of the CD11b positive human peripheral blood T lymphocytes coexpress the S-100 protein. These findings also demonstrate that S-100 positive T lymphocytes are restricted to a discrete T cell compartment which shows the TB positive CD11b phenotype.

These data open up an interesting problem in the scope of a recent topic on neuroendocrine and immune system interaction. As already shown (Blalock, 1988) the cells of the immune system, including gut associated lymphoid tissue (GALT), have potential to produce a variety of peptide hormones and express receptors for these peptides.

Materials and Methods

The pylorus and proximal duodenum were removed during the pathological section of twenty human fetuses with gestational ages of 18 to 42 weeks. Fetuses were obtained from spontaneous abortions (n=11), premature births (n=8) and prolonged pregnancy (n=1). In addition, specimens from two infants of 6 and 8 week old were included in this study.

Tissue specimens were fixed in 4% phosphate buffered formaldehyde (pH 7.2) and in Bouin's fluid. After dehydration the material was embedded in paraffin and sectioned at 8±1 µm. After de-waxing and rehydration, sections were stained according to Grimelius (1968) and used for immunohistochemistry. The peroxidase-antiperoxidase (PAP) technique, as described by Sternberger (1979), was then applied. The PAP complex was visualized with 0.05% 3.3-diaminobenzidine tetrachloride (DAB) dissolved in phosphate buffered saline (PBS) (pH 7.6) or 3-amino-9-ethylcarbazol (AEC) in 0.05 M acetate buffer (pH 5.2), containing 0.5% H_2O_2 at room temperature (5 minutes with DAB and 45 minutes with AEC). Thorough rinsing in PBS was carried out between different steps which were performed in a moist chamber. The primary antisera (from «Dacopatts») were used at the optimal dilutions in PBS for gastrin (1:2000), somatostatin (1:250), NSE (1:200) and S-100 protein (1:100). To test the specificity of immunoreactions the following controls were carried out: replacement of the primary antiserum by nonimmune rabbit or swine serum and by omission of single steps of the PAP-procedure. For gastrin immunoreaction, additional experiments were performed by modulating ionic strength, molarity and pH of PBS.

For the quantitative analysis of neuroendocrine cells a stereological method based on the point-counting principle was employed. Using a light microscope («Leitz») with a lens magnification of x40, endocrine cells were analyzed in terms of their volume density (V_V). An eyepiece (x10) with Weibel's multipurpose test system (M42) served for point counting. For the V_V (mm°) of G-cells, D-cells and NSE-positive cells, 50 to 160 fields of views («n») were calculated for counting according to De Hoof's formula (Weibel, 1979). In each field test points falling on selective stained endocrine cells and enterocytes were counted. Twelve serial sections were considered suitable for stereological analysis if they consisted of the full thickness of the mucosa and were cut perpendicular to the surface.

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