Virchows Arch [Pathol Anat] (1983) 400:163–171

Virchows Archiv A

Simultaneous immunohistochemical localization of gastrin releasing peptide (GRP) and calcitonin (CT) in human bronchial endocrine-type cells

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Summary. Formalin-fixed paraffin-embedded sections of fetal and adult human lungs were examined for the localization of gastrin releasing peptide (GRP) and calcitonin (CT) in bronchial endocrine-type cells with the indirect immunoperoxidase method. In fetal lungs, the appearance of CT was much later than that of GRP, and CT-containing cells were less frequent than GRP-containing cells which, in later fetal life, formed "neuroepithelial bodies (NEB)". NEB revealed little CT immunoreactivity. The serial section technique demonstrated that all CT immunoreactants in fetal and neonatal lungs were present within GRP-containing cells. An increase of CT immunoreactivity in GRP-containing cells was observed in perinatal lungs. The lung of a neonate who died of hyaline membrane disease contained the most abundant CT immunoreactants. In adult lungs, CT immunoreactivity was identified in some GRP-containing cells but cells containing only GRP or CT were also present. Cells containing both hormones occasionally formed hyperplastic foci in the bronchi of fibrotic lungs. Most cells of pulmonary tumorlets consistently showed GRP immunoreactivity, but the number of CT immunoreactive cells in them varied greatly.

Key words: Immunoperoxidase methods – Gastrin releasing peptide – Calcitonin – Human lung – Pulmonary tumorlet

Introduction

Several kinds of peptide hormone-containing endocrine-type cells have been identified in normal human bronchial mucosa. Bombesin immunoreactivity was first detected by Wharton et al. (1978) in human fetal lungs, but it was not found in adult lungs. Calcitonin (CT)-containing cells have been immunohistochemically demonstrated in human neonates by Becker et al.

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(1980). Cutz et al. (1981) have showed the presence of bombesin, CT and leu-enkephalin immunoreactivities in normal human lungs throughout life. Recently, we have reported that cells containing gastrin releasing peptide (GRP), a mammalian counterpart of amphibian bombesin, are present in human fetal as well as adult lungs, and that "neuroepithelial bodies (NEB)" in fetal lungs consistently show GRP immunoreactivity (Tsutsumi et al. 1982; 1983). We have also contended that in fibrotic lungs GRP-containing cells sometimes form hyperplastic foci, where ACTH-containing cells can also be infrequently identified (Tsutsumi et al. 1982; 1983).

In this article, we describe an immunohistochemical proof for the co-existence of GRP and CT immunoreactivities in the same bronchial endocrine-type cells in fetal and adult human lungs. The physiological, molecular biological and pathological significance of the concomitant localization is discussed.

Materials and Methods

Patients. All lungs obtained at autopsy or by therapeutic abortion were routinely fixed in 10–20% formalin for 1–7 days. One sample was taken and embedded in paraffin. The lungs examined were from 7 stillborn fetuses (12, 21, 22, 28, 34, 40 and 41 weeks gestation), 5 postnatal deaths, 3 dying of congenital disorders (7 days, 2 months and 6 months), 1 of intussusception (7 months) and 1 of hyaline membrane disease (24 days). Thirteen normal adults dying of non-pulmonary disorders (aged 31–88 years, mean 62.5) were examined, together with 10 adults with pulmonary disorders with fibrosis (aged 27–87 years, mean 64.8) and 6 adults with microscopic pulmonary tumorlets in the vicinity of fibrotic lesions (aged 60–77 years, mean 70.8).

Antisera and antigens. Two lots of rabbit anti-porcine GRP antisera, R-6902 and R-6903, were used for immunohistochemical staining at a 1:500–2,000 dilution. Preparation and characterization of these antisera has been described elsewhere (Yanaihara et al. 1981). Regarding the region-specificities, antiserum R-6902 recognizes two different antigenic determinants present at the middle and C-terminal portions of the porcine GRP sequence, while antiserum R-6903 is specific for the C-terminal portion of porcine GRP with a negligible cross-reactivity to substance P by the radioimmunoassay (Yanaihara et al. 1981). Rabbit anti-human CT antiserum was purchased from Immulok Inc., USA and used for immunostaining at a 1:4 dilution. Anti-substance P antiserum R-2404 was raised in a rabbit as described previously (Yanaihara et al. 1977) and used for immunostaining at a 1:500 dilution. Horseradish peroxidase-labeled goat IgG Fab fragment against rabbit IgG was prepared in our laboratory. Synthetic porcine GRP was prepared as described previously (Mochizuki et al. 1981). Synthetic human CT was purchased from Protein Research Foundation, Japan.

Immunohistochemistry. The indirect immunoperoxidase method after Nakane was applied for the detection of GRP and CT in the specimens. Deparaffinized sections were oxidized in 0.5% periodic acid for 10 min to remove the endogenous peroxidase activity, which was further suppressed by adding 0.01 M sodium azide in 0.01 M Tris-HCl buffer at pH 7.6 containing 30 mg/dl 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals, Japan) and 0.01 M hydrogen peroxide. Incubation time for the primary and secondary antibodies was 30 min, and washing of the unreacted antisera was performed for 15 min with 0.01 M phosphate buffered saline at pH 7.4. Nuclear counterstaining was done with 1% methylgreen at pH 4.0.

The specificities of the respective immunostainings were examined by the immunoabsorbance experiments. An excess (5 or 50 μg/ml) of synthetic porcine GRP or synthetic human CT was preincubated with the above diluted antisera at 37° C for 1 h, and the absorbed antisera were used for control immunostainings. The primary antiserum was also replaced

by anti-substance *P* antiserum to rule out the crossreactivity of the GRP antisera to substance *P*. Substance *P* antiserum could also serve as a negative control (an indifferent rabbit antiserum).

In order to investigate whether the same cells contain different peptides, two or more consecutive sections were stained with anti-GRP or anti-CT antiserum.

Results

It was clear from the serial section technique that both GRP antisera, R-6902 and R-6903, recognized the same cells. The specificities of positive immunostainings of GRP and CT described below were confirmed by the immuno-

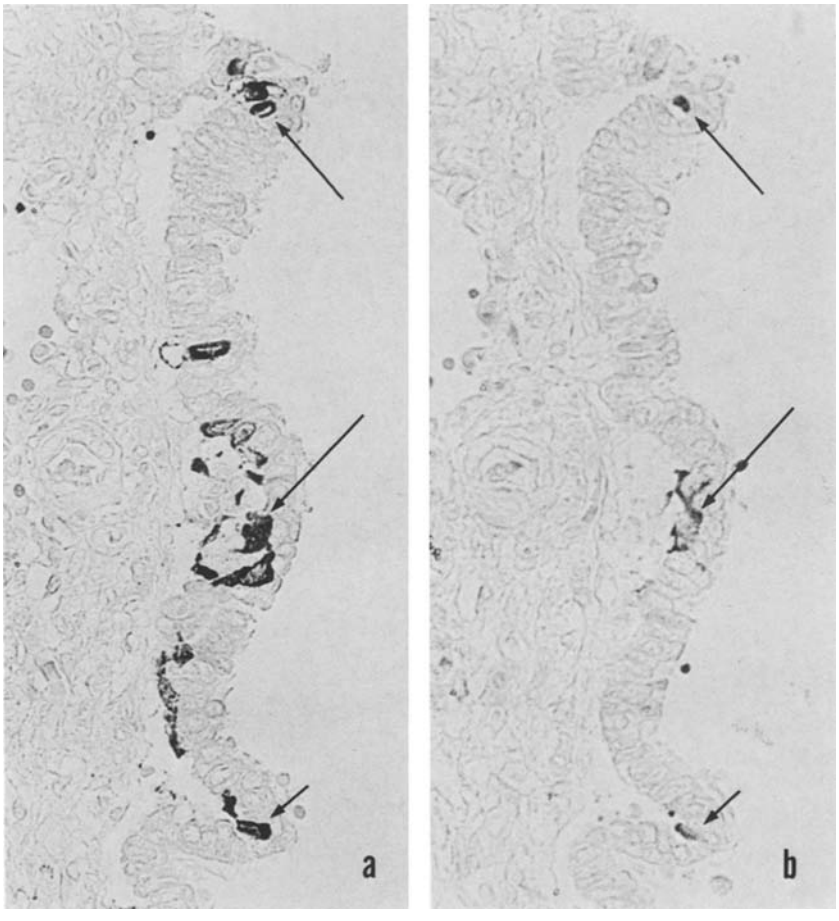

Fig. 1. Serial sections (**a**, **b**) prepared from the lung of a 7-day-old newborn infant dying of congenital malformation of the heart. Indirect immunoperoxidase staining for GRP (R-6903) (**a**) and CT (**b**). ×450. GRP-containing cells (**a**) are more numerous than CT-containing cells (**b**), and all CT immunoreactants are detected in a subpopulation of the GRP-containing cells (*arrows*)

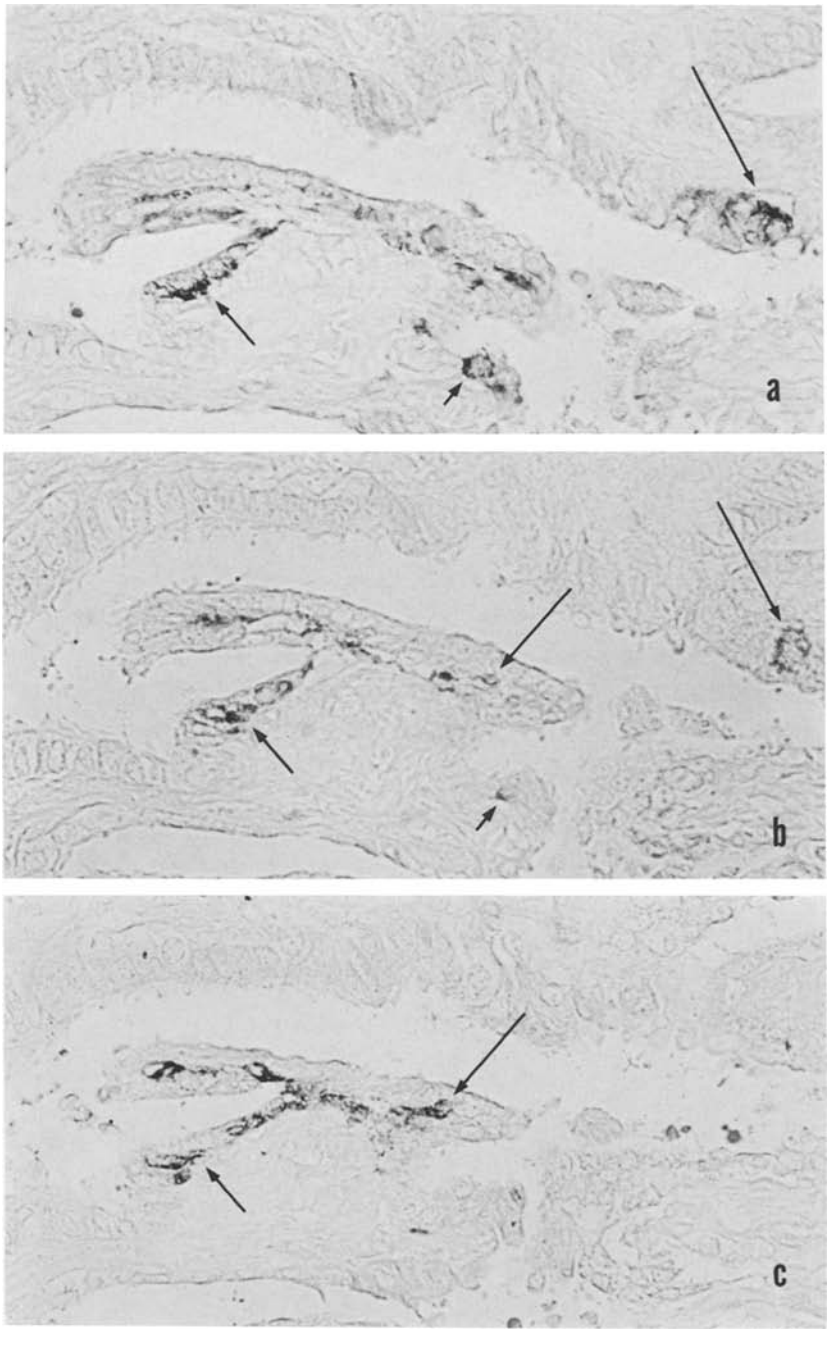

Fig. 2. Three serial sections (**a**, **b**, **c**) prepared from the lung of a 50-year-old female suffering from organized pneumonia with fibrosis. Indirect immunoperoxidase staining for GRP (**a**: R-6902, **c**: R-6903) and CT (**b**). ×420. A bronchiole surrounded by fibrosis shows focal hyperplasia of endocrine-type cells containing both GRP (**a** and **c**) and CT (**b**) (*arrows*)

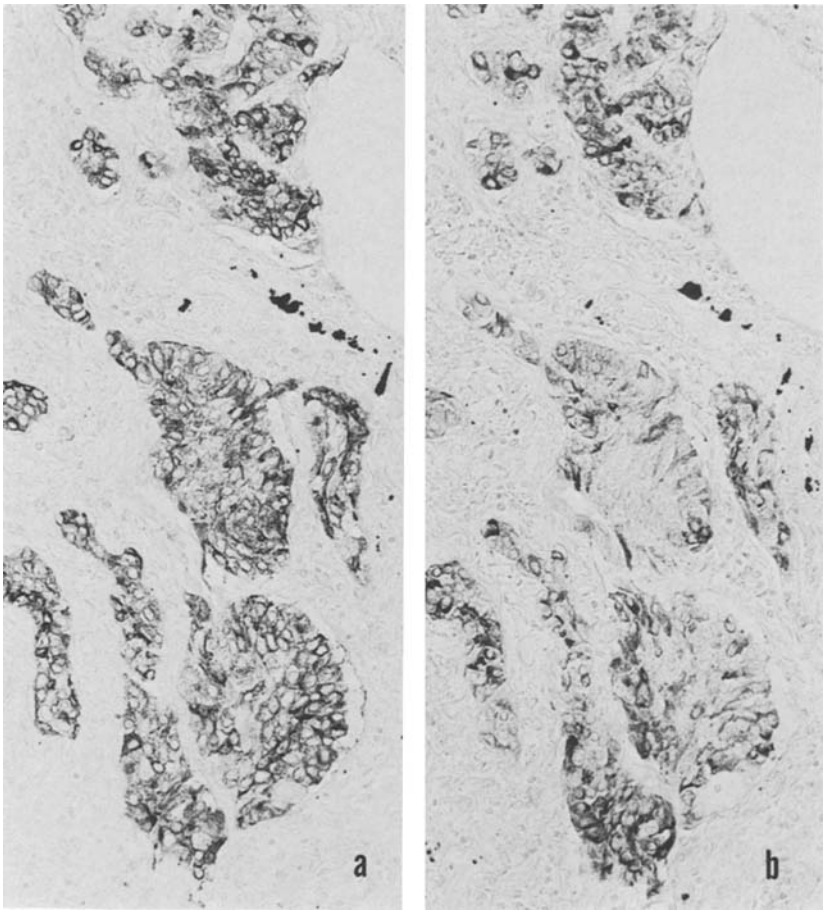

Fig. 3. Serial sections (**a**, **b**) of pulmonary tumorlet occurring in a focal scar from a 75-year-old female. Indirect immunoperoxidase staining for GRP (R-6903) (**a**) and CT (**b**). ×240. Most of the tumorlet cells located in the fibrotic interstitium exhibit both GRP (**a**) and CT (**b**) immunoreactivities

absorption tests as follows. Cells reactive with both GRP antisera were negative when 5 μg/ml of synthetic porcine GRP was added to the antisera. However, 50 μg/ml of synthetic human CT failed to abolish the positive reaction for GRP. Cells reactive with CT antiserum were negative only when 5 μg/ml of synthetic human CT was preincubated with the antiserum; 50 μg/ml of synthetic porcine GRP failed to abolish the CT immunostaining. No positive cells were found in the specimens with substance *P* antiserum.

In early-developing fetal lungs (12, 21 and 22 fetal weeks), there were a small number of GRP-containing cells along the basal part of the immature bronchi, but no CT-containing cells were identified. Later in fetal life (28, 34, 40 and 41 fetal weeks), the number of GRP-containing cells in bronchial mucosa was gradually increased and the formation of "neuroepithelial bodies (NEB)" by GRP-containing cells was frequently noted. At

this stage of development, a few isolated CT-containing cells appeared, but CT immunoreactivity was marginal in NEB. That is, the number of CT-containing cells, which slightly increased in lungs of a fetus aged 41 weeks, was much less than that of GRP-containing cells. Furthermore, CT immunoreactivity was always detected in the cells containing GRP. Postnatally, the number of GRP-containing cells remained fairly high until 2 months, but was markedly decreased in lungs of 6- and 7-month-old infants. CT-containing cells increased in number in newborn lungs (7 days and 24 days). These CT immunoreactants were again exclusively detected in GRP-containing cells (Fig. 1). The lung of a 24-day-old neonate who suffered from hyaline membrane disease contained especially plentiful CT-containing cells. Lungs of older infants (2–7 months old) showed a reduced CT-containing cell number. In normal adult lungs, a few cells containing GRP or CT were usually present, except for one lung: Isolated cells were distributed in the mucosa of small bronchi or bronchioles. The number of GRP-containing cells and of CT-containing cells were roughly equal in the adult lungs. GRP and CT were occasionally identified within the same endocrine-type cells, although cells containing either GRP or CT separately were also present. Fibrosing adult lungs also showed a few isolated GRP- and/or CT-containing cells, and 4 of the lungs revealed focal GRP-cell hyperplasia. Three of 4 such hyperplastic lesions exhibited the co-existence of CT immunoreactants in the increased GRP-containing cells (Fig. 2). However, the other GRP-cell hyperplastic focus contained only a few CT-immunoreactive cells. Almost all cells consisting in pulmonary tumorlets (6 lesions examined) showed GRP immunoreactivity, while the number of cells containing CT immunoreactivity varied from many (2 lesions), moderate numbers (2 lesions) or a few (2 lesions). Figure 3 shows a tumorlet lesion lying in the anthracotic fibrous interstitium and most of its constituents revealed both GRP and CT immunoreactivities simultaneously.

Discussion

The presence of bombesin and calcitonin (CT) immunoreactive endocrine-type cells in human bronchial mucosa has been previously demonstrated (Wharton et al. 1978; Becker et al. 1980; Cutz et al. 1981). Whether these hormones are localized in different cells has not yet been clarified. Bombesin and smaller numbers of CT immunoreactants have been detected in neuroepithelial bodies (NEB) (Cutz et al. 1981) which have been postulated to be hypoxia-sensitive chemoreceptors (Lauweryns et al. 1978). Recently, we have described the occurrence of immunoreactivity of gastrin releasing peptide (GRP), which is a mammalian counterpart of amphibian bombesin and has a very similar C-terminal amino acid sequence to bombesin (McDonald 1981), in human bronchial endocrine-type cells and in NEB (Tsutsumi et al. 1982; 1983). A bombesin-specific antiserum which is not cross-reactive to GRP has failed to demonstrate any bombesin immunoreactant in human lungs immunohistochemically (Tsutsumi et al. 1983). We have further shown that GRP-containing cells may form hyperplastic foci in fibrosing lungs and that GRP immunoreactivity is consistently detected

in most cells of pulmonary tumorlets. It is frequently found in some cells of bronchial carcinoids or small cell lung carcinomas, especially the intermediate cell type (Tsutsumi et al. 1982; 1983). It has also been pointed out that ACTH-containing cells are present in these GRP-cell hyperplastic or neoplastic lesions, but the cells containing GRP and ACTH are not identical. (Tsutsumi et al. 1982; 1983).

In this report, we present evidence that all CT immunoreactants in human fetal and neonatal lungs and some CT immunoreactants in human adult lungs co-exist within a subpopulation of the GRP-containing cells. The absence of cross-reactivities of both antisera in the immunostainings was confirmed by the immunoabsorbance experiments with synthetic porcine GRP and synthetic human CT. Regarding GRP, we have previously reported that most of the GRP immunoreactants in human lungs largely belong to the bioactive C-terminal portion of GRP (Tsutsumi et al. 1983).

Simultaneous localization of two different peptide hormones in a single cell is rare except for those cases in which they evidently share a common precursor molecule. Such rare instances include 1) (entero)glucagon and bovine pancreatic polypeptide (BPP) in human colonic endocrine cells (Lehy et al. 1981) and in gastric A cells of human fetal oxyntic mucosa (Tsutsumi, unpublished data), 2) CT and somatostatin in rat and rabbit thyroid C-cells (Alumets et al. 1980; Buffa et al. 1979), and 3) interestingly enough, GRP and CT in reactively hyperplastic human thyroid C-cells in patients with hypercalcemia (Kameya et al. 1983). The explanation for the co-existence of two hormones in one cell is controvertial, but is seemingly very important to understand physiological roles of the hormones.

Relevant explanations for the co-existence of GRP and CT in single bronchial endocrine-type cells are as follows. Firstly, cross-reactivity of CT antiserum to the precursor molecule of GRP or vice versa should be mentioned. Cross-reactivity of CT antiserum to the 31 K precursor molecule giving rise to ACTH, β-endorphin and other related peptides in rat pituitary intermediate lobe has been reported (Deftos et al. 1978). The presence of gastric inhibitory peptide, cholecystokinin or β-endorphin immunoreactivity in pancreatic islet A-cells has been interpreted as the cross-reactivity of the antibodies to the proglucagon molecule (Patzelt et al. 1979). A similar notion has been proposed concerning the presence of BPP immunoreactivity in colonic enteroglucagon cells (Buffa et al. 1979). Processing mechanisms of the CT gene have been clearly and precisely analysed at the molecular biology level and the absence of a homologous sequence with porcine GRP in any part of the amino acid sequence corresponding to the CT gene has already been clarified (Amara et al. 1982). Yet little is known about a precursor molecule of GRP. In this context, the possibility of cross-reaction of the antisera to precursor molecules remains undetermined.

The second possibility that GRP and CT share a common precursor molecule would rather be unlikely because of the findings described above (Amara et al. 1982).

Thirdly, it is conceivable that GRP and CT would be encoded in closely linked but independent genes, and their expression is controlled by coupled regulatory mechanisms, as is the case with alpha-fetoprotein and albumin

in mammalian liver cells (Tilghman and Belayew 1982) and delta and mu chains in mouse lymphocytes (Maki et al. 1981). The mode of these gene expressions could be influenced by various environmental conditions under development or in diseases (i.e. hyaline membrane disease, lung fibrosis and pulmonary tumorlet).

Forthly, Alumets et al. (1980) have suggested that somatostatin-containing cells in rat thyroid are capable of taking up CT from the environment and accumulating it intracellularly. In this regard, identity or non-identity of the intracellular localization of GRP and CT in a single cell should be investigated at the ultrastructural level.

True control mechanisms on the expression or secretion of GRP and CT in a single cell must be determined by further detailed investigations at the molecular biology level.

Regarding the functions of GRP and CT in the lung, little is known to date. GRP stimulates 1) pancreatic enzyme secretion, 2) gallbladder contraction, 3) vasocontraction and 4) secretion of gastrin, insulin and enteroglucagon as does bombesin (McDonald 1981). Bombesin further functions as a bronchoconstrictor (Impicciatore and Bertaccini 1973) and as a growth stimulator on in vitro human small cell lung cancer cell lines (Minna et al. 1982). Functions of CT other than a calcium-lowering effect have yet to be established. The wide distribution of GRP in central and autonomic nerves (Yanaihara et al. 1981) and the demonstration of CT immunoreactivity in frog and human brain (Yui et al. 1981; Becker et al. 1979) suggest that GRP and CT may function as a neurotransmitter or neuromodulator. In the lung, we should pay attention to their roles specific to the organ. Clues may lie in the present immunohistochemical observations in several pathological conditions. The number of cells containing both GRP and CT is most abundant in the lung of a neonate who died of hyaline membrane disease. Fibrosing lung diseases occasionally induce focal hyperplasia of endocrine-type cells in diseased bronchial mucosa and these hyperplastic lesions consist of cells containing both GRP and CT or cells containing GRP and far less CT immunoreactants. Similar observations are obtained regarding the pulmonary tumorlets whose true nature has not yet been settled. These pathological changes should always be referred to physiological/ontogenetical changes. Possible kinetic changes or changes in hormone expression in bronchial endocrine-type cells, accompanied by normal development or pathological processes, require further investigation in order that we may understand the physiology and pathophysiology of the lung from an endocrinological point of view.

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Accepted March 2, 1983