Prognostic Implications Of Calbindin-D28k Expression in Lung Cancer: Analysis of 452 Cases

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Calbindin D28k (Ca-D28k) acts as a buffering system to maintain cellular calcium homeostasis and is thought to play a role in inhibiting apoptosis. The goals of this study were to assess CA-D28k expression in lung carcinomas and to correlate these results with patient survival.

A total of 452 lung carcinomas were immunostained with a monoclonal antibody specific for Ca-D28K using an avidin-biotin peroxidase technique. The number of cells with nuclear staining was graded semiquantitatively into one of five groups: 0, fewer than 10%, 10 to 25%, more than 25 to 50%, more than 50 to 75%, and more than 75%. Results were correlated with patient survival using Kaplan-Meier survival curves.

A total of 335 of 452 (74%) lung carcinomas were positive for Ca-D28k. There was no statistically significant difference in the prevalence of Ca-D28k expression in tumors of different histologic type. Kaplan-Meier survival analysis revealed that for patients with adenocarcinoma, those with Ca-D28k-positive tumors had a better overall survival than patients with Ca-D28k-negative tumors ($P = .036$). This difference was also significant for patients with Stages I and II adenocarcinomas ($P = .033$). No statistically significant difference in prognosis was observed for patients with Stages III and IV adenocarcinomas or for patients with other lung carcinoma types of varying stage.

Ca-D28k is commonly expressed in lung carcinomas of all histologic types. For patients with localized adenocarcinoma of the lung, Ca-D28k expression correlated with improved survival. No correlation between Ca-D28k expression and patient survival was found for disseminated adenocarcinoma and for other histologic types of lung carcinoma.

KEY WORDS: Calbindin D28k, Immunohistochemistry, Lung carcinoma.

Since its initial discovery by Waisserman and Taylor in 1966, calbindin has been reported in a wide variety of species and tissues, including cerebellum, kidney, placenta, thymus gland, and neuroendocrine cells of the gastrointestinal tract, lung, pancreas, and thyroid gland (1–6). Calbindin belongs to a family of intracellular proteins that have affinity for calcium. Other family members include S-100 protein, calmodulin, parvalbumin, and troponin C (7). Calbindins are acidic proteins that are heat stable. Two major subclasses have been described: D9k and D28k. Calbindin D28k is encoded by a gene located on chromosome 8 that is highly conserved in evolution (7). Ca-D28k is a single polypeptide chain that consists of 261 amino acid residues, with four high-affinity calcium-binding sites.

The physiologic function of Ca-D28k is unknown, although it has been postulated that Ca-D28k (and other calbindins) constitutes an intracellular buffering mechanism to adjust calcium to a physiologic level (7). Because calcium is thought to play an important role in apoptosis by stimulating calcium/magnesium-dependent endonucleases and by modulating calcium/calmodulin-dependent enzymatic activity (8), Ca-D28k may play a role in modulating apoptosis. Dowd and colleagues (8), using a thymoma cell line, showed that Ca-D28k overexpression decreases sensitivity to apoptosis, presumably by interfering with calcium fluxes that induce programmed cell death.

Previous studies of Ca-D28k expression in lung cancer have been few and limited in scope (9), and the prognostic relevance of Ca-D28k expression in lung cancer has not been examined. The purpose of

this study was to assess the prevalence of Ca-D28k expression in lung carcinomas of various histologic types and to determine whether Ca-D28k expression has any prognostic significance.

MATERIALS AND METHODS

Paraffin blocks of formalin-fixed biopsy and surgical resection tissues were obtained from the archives of The Methodist Hospital, Houston, Texas. A representative paraffin block of each neoplasm was selected. The histologic classification of each tumor was based on the World Health Organization criteria (10). Stage was determined using the TNM system (11).

Medical charts and cancer registry data for each patient were reviewed for age, sex, smoking history, clinical and pathologic staging, and survival. Survival was correlated with age, sex, histologic pattern of carcinoma, stage, and presence or absence of Ca-D28k immunostaining. The minimum follow-up period for each surviving patient was at least 5 years. Patients who died within 3 months after surgical excision or who died from causes other than lung cancer within 5 years after surgery were excluded from the final statistical analysis.

Immunohistochemical staining for Ca-D28k was done using routinely processed, paraffin-embedded tissue sections. Briefly, after slides were treated with hydrogen peroxide to block endogenous peroxidase activity, they were washed in distilled water and immersed in 0.01 M sodium citrate buffer (pH 6.0) for 20 min at 95° C, followed by rinsing in distilled water and phosphate-buffered saline. The slides were then incubated for 2 h with a monoclonal antibody specific for Ca-D28k (dilution 1:250, code no. 300; Swant Swiss Antibodies, Bellinzon, Switzerland). Staining for Ca-D28k was carried out with an avidin-biotinylated peroxidase method according to the recommendations of the manufacturer (Vector Laboratories, Burlingame, CA). A lung carcinoma was considered to be positive for Ca-D28k if at least 10% of the tumor cells exhibited nuclear staining. The number of positive tumor cells was graded semiquantitatively using the following five groups: 0, less than 10%, more than 10 to 25%, more than 25 to 50%, more than 50 to 75%, and more than 75%. All stained cells were recorded as positive regardless of staining intensity.

Overall patient survival was calculated from the date of operation by Kaplan-Meier cumulative survival plot using SPSS 7.5 software (Chicago, IL). Overall survival was compared across levels of prognostic factors using the log-rank test. All statistical tests were two sided.

RESULTS

A total of 452 patients with primary lung carcinoma diagnosed between 1984 and 1991 were studied. The demographic characteristics, smoking history, and survival data are shown in Table 1. A total of 425 patients (94%) had a positive history of smoking. A total of 151 patients (33%) were alive 5 years after diagnosis, with a mean follow-up period of 44 months.

The lung carcinomas assessed in this study were as follows: 178 (39%) adenocarcinoma, 113 (25%) squamous cell, 73 (16%) large cell undifferentiated, 62 (14%) bronchioloalveolar, 17 (4%) small cell, and 9 (2%) adenosquamous. The distribution of stage, treatment and mean survival is shown in Table 2. The mean survival was best for patients with bronchioloalveolar carcinoma (63 months) and worst for patients with small cell carcinoma (29.8 months).

Table 3 summarizes the frequency of Ca-D28k expression for the different types of lung carcinoma. A total of 335 of the 452 (74%) lung carcinomas were positive for Ca-D28k (Fig. [fgc]l). There was no difference in Ca-D28k immunostaining between biopsy and resection specimens. There was no significant correlation between the prevalence of Ca-D28k expression and histologic type of carcinoma. The prevalence of immunohistochemically detectable Ca-D28k in each tumor type was as follows: 8 (88%) adenosquamous carcinoma, 48 (77%)

TABLE 1. Histologic Type and Demographic Characteristics of 452 Patients with Lung Cancer

	SQC	AD	ADSQC	BA	LC	SC	Total
N	113	178	9	62	73	17	452
Age (mean y)	66	63	64	64	64	66	64
Sex							
Male	85	104	8	29	46	10	282 (62%)
Female	28	74	1	33	27	7	170 (38%)
Smoking history							
Never	1	12	2	11	5	0	31 (7%)
Current/recent	89	145	6	42	63	17	362 (81%)
Quit >1 y	22	18	1	7	5	6	59 (12%)
Survival							
Alive	28	55	3	30	20	15	151 (33%)
Dead	85	123	6	32	53	2	301 (67%)

SQC, squamous cell carcinoma; AD, adenocarcinoma; ADSQC, adenosquamous cell carcinoma; BA, bronchioloalveolar carcinoma; LC, large cell carcinoma; SC, small cell carcinoma.
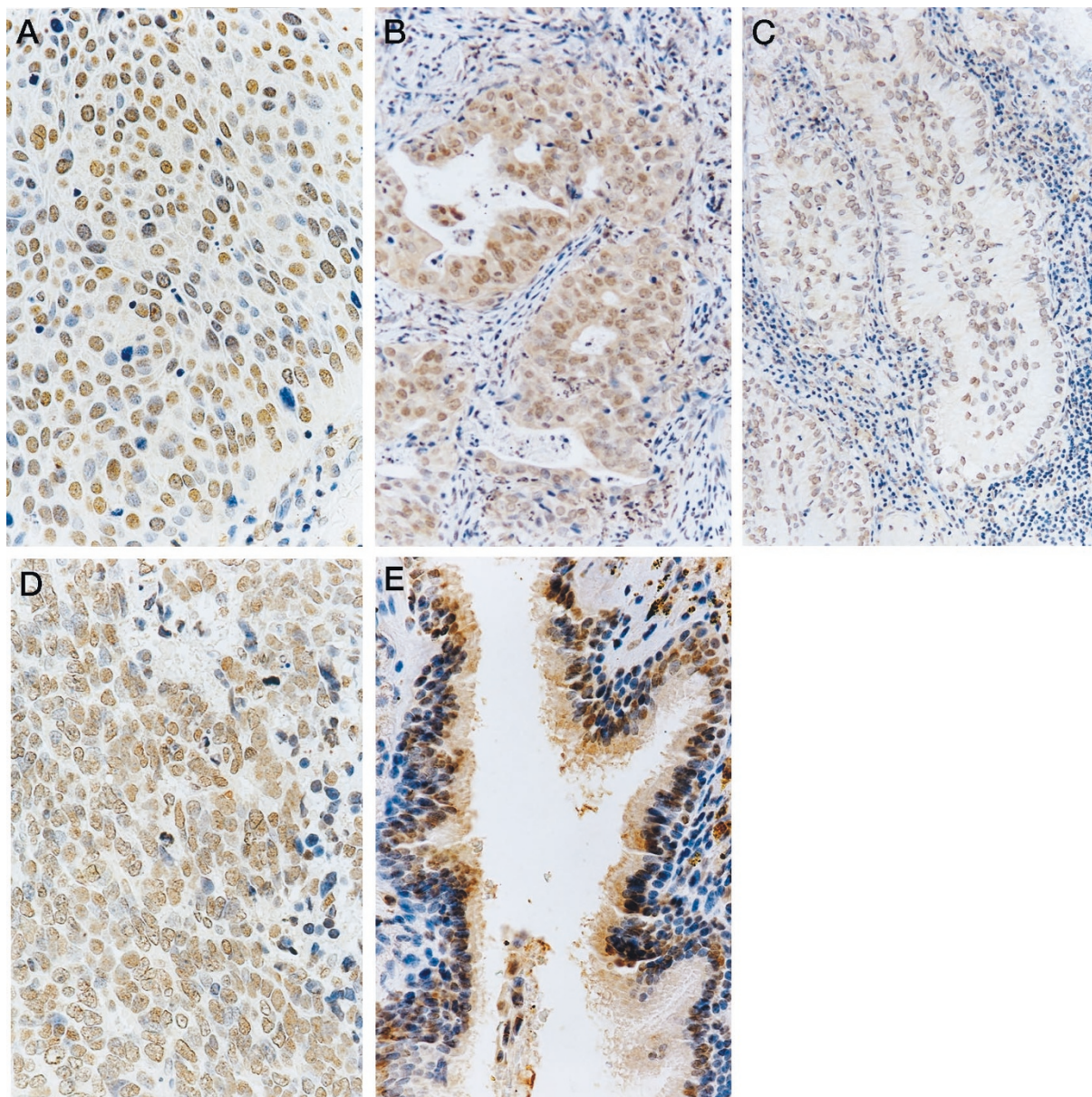
FIGURE 1. Nuclear and cytoplasmic expression of Ca-D28k. **A**, squamous cell carcinoma; **B**, adenocarcinoma; **C**, bronchioloalveolar carcinoma; **D**, small cell carcinoma; **E**, normal bronchial epithelium.

bronchioloalveolar carcinoma, 86 (76%) squamous cell, 13 (76%) small cell carcinoma, 135 (75%) adenocarcinoma, and 45 (61%) large cell undifferentiated carcinoma.

The mean survival for Ca-D28k–positive tumors (49 months) was slightly better than for Ca-D28k–negative tumors (45 months), with a value approaching statistical significance (Table 3; $P =$ .061). Ca-D28k expression significantly correlated with survival for patients with adenocarcinoma. Patients with Ca-D28k–positive tumors (mean, 44.1 months) had improved survival over patients with Ca-D28k–negative tumors (mean, 29.2 months; $P =$ .036) Table 3 and Fig. 2. This was also true for patients with Stages I and II adenocarcinoma ($P =$ .033), but not for patients with Stages III and IV

adenocarcinoma. There were no other statistically significant differences in patient survival between Ca-D28k–positive and Ca-D28k–negative lung tumors of other histologic types.

Ca-D28k expression was analyzed in relation to various clinical characteristics including sex, smoking history, TNM stage, and therapy (Table 4). None of these characteristics correlated with Ca-D28k expression.

DISCUSSION

Previous studies of Ca-D28k expression assessed in lung cancer have been limited in scope (9). In this study, we assessed Ca-D28k expression in a

TABLE 2. Histologic Type, Pathologic Stage, Treatment, and Survival of 452 Patients with Lung Cancer

|	SQC	AD	ADSQC	BA	LC	SC	Total
N	113	178	9	62	73	17	452
Stage							
I	64	114	5	52	44	10	289 (64%)
II	22	36	2	7	12	3	82 (18%)
III	16	20	1	3	7	1	48 (11%)
IV	11	8	1	0	10	3	33 (7%)
Treatment							
Surgery	71	113	7	48	42	5	286 (64%)
Surgery + RT/CT	38	63	1	12	21	10	145 (32%)
RT/CT	3	2	1	2	10	2	20 (4%)
No treatment	1	0	0	0	0	0	1 (.2%)
Survival (mean, mo)	44.3	47.7	46.2	63	45.9	29.8	47.7
Follow-up (mean, mo)	84.7	81	79.7	81.2	92.6	69.2	83.3

SQC, squamous cell carcinoma; AD, adenocarcinoma; ADSQC, adenosquamous cell carcinoma; BA, bronchioloalveolar carcinoma; LC, large cell carcinoma; SC, small cell carcinoma; RT, radiation therapy; CT, chemotherapy.

TABLE 3. Histologic Type of Lung Cancer, Ca-D28k Expression, and Patient Survival

Histologic Type	Ca-D28k Positive		Ca-D28k Negative		p^a
	Prevalence	Survival (mean, mo)	Prevalence	Survival (mean, mo)	
SQC	86 (76%)	30.3	27 (24%)	34.5	0.808
AD	135 (75%)	44.1	43 (25%)	29.2	0.036
ADSQC	8 (88%)	20.4	1 (12%)	50	0.748
BA	48 (77%)	99.6	14 (23%)	63.9	0.241
LC	45 (61%)	31.0	28 (39%)	32.53	0.673
SC	13 (76%)	24.8	4 (24%)	19.9	0.091
Total	335	49	117	45	0.061

SQC, squamous cell carcinoma; AD, adenocarcinoma; ADSQC, adenosquamous cell carcinoma; BA, bronchioloalveolar carcinoma; LC, large cell undifferentiated carcinoma; SC, small cell carcinoma.

^{a} *p* value refers to difference in survival between patients with Ca-D28k–positive and Ca-D28k-negative tumors according to histologic type.
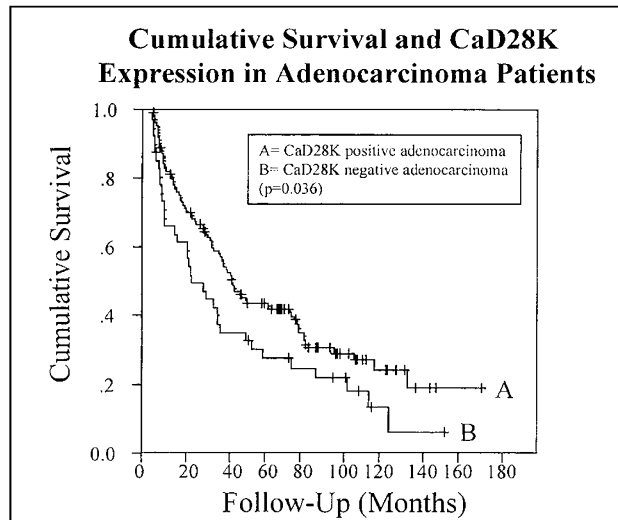
FIGURE 2. Kaplan-Meier survival curves for patients with lung adenocarcinoma.

large number of lung carcinomas of various histologic types and have compared Ca-D28k expression with patient demographic characteristics and survival.

We evaluated Ca-D28k expression using an immunohistochemical method and detected Ca-D28k expression in 335 of 452 (74%) tumors. The prevalence of Ca-D28k expression was similar for all histologic types of lung carcinoma assessed: adenosquamous (88%), bronchioloalveolar (77%), squamous cell (76%), small cell (76%), adenocarcinoma (75%), and large cell undifferentiated (61%). These results are in agreement with those of Watanabe and colleagues (9), who measured the concentration of Ca-D28k in lung cancer cell lines using enzyme immunoassay methods. They reported Ca-D28k expression in all lung cancer cell lines; however, the prevalence of Ca-D28k expression was highest in small cell carcinoma. A possible explanation for the discrepant results between our study and Watanabe *et al.*'s (9) is the use of different detection methods or the different types of specimens assessed.

Our findings contrast with those of Katsetos and colleagues (12), who studied immunohistochemical expression of Ca-D28k in a small study of 41 lung tumors. In that series, Ca-D28k expression was observed only in neuroendocrine tumors, including four small cell carcinomas, two atypical carcinoids, and two typical carcinoids assessed. Katsetos *et al.* (12) did not demonstrate Ca-D28k in other lung carcinoma types. Other reports also have found that Ca-D28k is expressed in normal endocrine cells of stomach (4, 5), respiratory epithelium (13), pancreas (14), duodenum (15), and neuroendocrine tumors of the gastrointestinal tract (12). Thus, others have suggested that Ca-D28k may be a useful

TABLE 4. Demographic Characteristics of 452 Patients with Lung Cancer Positive or Negative for Ca-D28k

Variable	Ca-D28k+	Ca-D28k−	p Value
N	335	117	
Survival			
Alive	109	27	0.061
Dead	226	90	
Age (mean)	65	64	0.952
Sex			
Male	209	73	1.00
Female	126	44	
Smoking history			
Nonsmokers	25	8	0.662
Smokers	310	108	
Histologic type			
SQC	86	27	
AD	135	43	
ADSQC	8	1	0.238
BA	48	14	
LC	45	28	
SC	13	4	
Stage			
I	218	71	
II	56	26	0.637
III	34	14	
IV	27	6	
Therapy			
Surgery only	212	74	
Surgery + RT/CT	103	42	
RT/CT	15	5	0.696
No treatment	1	1	

SQC, squamous cell carcinoma; AD, adenocarcinoma; ADSQC, adenosquamous cell carcinoma; BA, bronchioloalveolar carcinoma; LC, large cell undifferentiated carcinoma; SC, small cell carcinoma; RT, radiation therapy; CT, chemotherapy.

marker of neuroendocrine differentiation (12). In contrast, we observed that CaD28k is present in a wide variety of lung carcinomas of all histologic types. Our results demonstrate that Ca-D28k expression is not limited to neuroendocrine tumors and may have more widespread functional significance.

We analyzed the prognosis of patients with lung carcinoma and Ca-D28k expression. Patients with Ca-D28k–positive adenocarcinomas had significantly better survival (mean, 44.1 months) than patients Ca-D28k–negative adenocarcinomas (mean, 29.2 months; $P = .036$). The difference in survival was also significant for patients with localized (Stages I and II) adenocarcinomas ($P = .033$). Ca-D28k expression did not significantly correlate with patient survival for disseminated adenocarcinomas or other histologic types of lung carcinoma. In some ways, this result is counterintuitive. If Ca-D28k overexpression is thought to play a role in inhibiting apoptosis (8), one might suspect that Ca-D28k–positive tumors would have a worse prognosis. However, other factors are involved that might be more important in determining patient prognosis, for example, the primary treatment modality (surgical excision alone *versus* surgical excision and adjuvant therapy) and the adequacy of surgical excision margins.

The true functional role of Ca-D28k is not known. In addition to its role in inhibiting apoptosis, some observations suggest that Ca-D28k may be involved in the regulation of the secretory process (1, 7). Adenocarcinomas with preservation of the mucin secretion are better differentiated and have a better prognosis than poorly differentiated adenocarcinomas. It is therefore possible that Ca-D28k–positive adenocarcinomas represent a group of tumors that are better differentiated than Ca-D28k–negative adenocarcinomas, although we did not observe a clear correlation between Ca-D28k expression and differentiation histologically in this study.

In prior studies, conflicting results regarding the localization of Ca-D28k at the cellular level have been published. In some studies of other malignant neoplasms and normal tissues, Ca-D28k expression was thought to be restricted to the cell membrane (2–6). In contrast, others (12–14) have reported results similar to our own. We observed that Ca-D28k is expressed within the nuclei and cytoplasm of lung carcinoma cells of all histologic types and in normal bronchial epithelium. The difference in the distribution of Ca-D28k within the cell is most likely a manifestation of different roles that the protein has within cells.

REFERENCES

1. Walker SW. Calcium-binding proteins in man and other animals. J Clin Biochem Nutr 1988;4:1–18.
2. Opperman LA, Pettifor JM, Ross FP. Immunohistochemical localization of calbindins (28K and 9K) in the tissues of the baboon Papio ursinus. Anat Rec 1990;228:425–30.
3. Furness JB, Padbury RTA, Baimbridge KG, Skinner JM, Lawson DE. Calbindin immunoreactivity is a characteristic of enterochromaffin-like cells (ECL cells) of the human stomach. Histochemistry 1989;92:449–51.
4. Walters JR, Bishop AE, Facer P, Lawson EM, Rogers JH, Polak JM. Calretinin and calbindin-D28k immunoreactivity in the human gastrointestinal tract. Gastroenterology 1993;104:1381–9.
5. Johnson EW, Eller PM, Jafek BW. Protein gene product 9.5-like and calbindin-like immunoreactivity in the nasal respiratory mucosa of perinatal humans. Anat Rec 1997;247:38–45.
6. Abe H, Watanabe M, Kondo H. Transient appearance of Ca-binding protein (spot 35-calbindin) in bronchial epithelial cells, thyroid parafollicular cells, and thymic epithelial cells during the development of rats. Histochemistry 1992;97:155–60.
7. Christakos S, Gabrielides C, Rothen WB. Vitamin D-dependent calcium-binding proteins: chemistry, distribution, functional considerations, and molecular biology. Endocr Rev 1989;10:3–26.
8. Dowd DR, MacDonald PN, Komm BS, Haussler MR, Miesfeld RL. Stable expression of the calbindin-D28K complementary DNA interferes with the apoptotic pathway in lymphocytes. Mol Endocrinol 1992;6:1843–8.
9. Watanabe H, Imaizumi M, Ojika T, Abe T, Hida T, Kato K. Evaluation of biological characteristics of lung cancer by the human 28kDa vitamin D-dependent calcium binding protein, calbindin-D28k. Jpn J Clin Oncol 1994;24:121–7.

10. World Health Organization. The World Health Organization histological typing of lung tumors. Am J Clin Pathol 1982;77:123–36.
11. Bulzebruck H, Bopp R, Drings P, Bauer E, Krysa S, Probst G, et al. New aspects in the staging of lung cancer. Prospective validation of the International Union Against Cancer TNM Classification. Cancer 1992;70:1102–10.
12. Katsetos CD, Jami MM, Krishna L, Jackson R, Patchefsky AS, Cooper HS. Novel immunohistochemical localization of 28,000 molecular-weight (Mr) calcium binding protein (calbindin-D28k) in enterochromaffin cells of the human appendix and neuroendocrine tumors (carcinoids and small-cell carcinomas) of the midgut and foregut. Arch Pathol Lab Med 1994;118:633–9.
13. Ito T, Udaka N, Inayama Y, Kitamura H, Kanisawa M. Hamster pulmonary endocrine cells with positive immunostaining for calbindin-D28k. Histochem Cell Biol 1998;109:67–73.
14. Roth J, Bonner-Weir S, Norman AW, Orci L. Immunocytochemistry of vitamin D-dependent calcium binding protein in chick pancreas: exclusive localization. Endocrinology 1982;110:2216–8.
15. Buffa R, Mare P, Salvadore M, Solcia E, Furness JB, Lawson DE. Calbindin 28 kDa in endocrine cells of known or putative calcium-regulating function. Thyro-parathyroid C cells, gastric ECL cells, intestinal secretin and enteroglucagon cells, pancreatic glucagon, insulin and PP cells, adrenal medullary NA cells and some pituitary (TSH?) cells. Histochemistry 1989;91:107–13.

Book Review

Markell EK, John DT, Krotoski WA: Markell and Voge's Medical Parasitology, 8th Ed, 501 pp, Philadelphia, W.B. Saunders, 1999 ($51).

This is the eighth edition of the classical text of parasitology first published in 1958. Over the years, the text has been updated periodically and the latest revision, finished in 1998 (almost coincidentally with Dr. Markell's death), reflects well the latest advances in this field.

The book consists of 16 chapters. The introductory general chapters are followed by those that discuss comprehensibly common diseases caused by protozoa, trematodes, nematodes, cestodes, arthropodes, and organisms of uncertain taxonomy. At the end is a chapter that provides an overview of parasitic diseases in high-risk populations, such as AIDS patients and immunocompromised hosts; a chapter that addresses signs and symptoms that are unique to some or common to more than one parasitic disease; and chapters that have detailed protocols for diagnostic procedures in clinical context.

Didactic line drawings summarize often the life cycle of various parasites, and color plates illustrate the most important diagnostic features of common parasites, larvae, and ova. The text contains numerous black and white photographs illustrating the clinically important aspects of parasitic diseases. Unfortunately, some of these photographs are not always informative and not of the highest quality. The authors would be well served to remove some of these or replace them with better photographs in the next edition. A list of parasitic diseases that must be reported by law in various states would also be welcome.

This is an excellent textbook for pathology residents and technicians and even clinical infectologists. Practicing pathologists could use it as a reference when dealing with parasitic diseases.

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