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Calcium-binding proteins in carcinoma, neuroblastoma and glioma cell lines

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Summary. Antisera against the Ca^{2+}-binding proteins parvalbumin, calbindin D-28K, and the S-100 proteins were used to study the distribution of their target proteins in selected human carcinoma (LICR-HN6;Caco-2), mouse neuroblastoma (clone NB-2a), and rat glioma cell lines (clone C-6). Pronounced staining with anti-parvalbumin was observed in the cytosol of all cells as well as in some nuclei, in particular, mitotic nuclei were highly immuno-reactive. Applying light and immune-electron microscopy (colloidal gold labelling) the parvalbumin-fluorescence was associated with filaments in the LICR-HN6 cells. However, this immunoreactivity was not a result of the presence of parvalbumin itself – as shown by biochemical analyses (HPLC, 2D-PAGE) – but was due to the presence of a Ca^{2+}-binding and tumour-associated protein with similar biochemical and immunological properties. S-100 proteins were present in all tumour cell lines but their intracellular distribution was different from calbindin D-28K. Calbindin-immunoreactivity was found on the membranes of the carcinoma cell lines whereas neuroblastoma and glioma cells remained unlabelled. It is suggested that these proteins might be involved in the modulation of the enhanced stimulation of Ca^{2+}-dependent processes occurring in tumour cells.

Key words: Ca^{2+}-binding proteins – Parvalbumin – Calbindin D-28K – S-100 – Tumour-associated protein – Human carcinoma cell lines – Mouse neuroblastoma – Rat glioma

Introduction

A variety of mammalian tumour cells display elevated levels of cytosolic Ca^{2+} (Tsuruo et al. 1984; Banyard and Tellam 1985; Whitfield et al. 1987) and Ca^{2+}-binding proteins (Chafouleas et al. 1981; Wei et al. 1982; Veigl et al. 1984; Zendegui et al. 1984) and this may be responsible in part for a permanent activation of DNA synthesis and/or their increased motility. 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2$] D_3 selectively inhibits proliferation and alters the morphology of certain tumour cell lines in vitro and furthermore, 1α,25$(OH)_2D_3$ receptors have been found in human solid tumours and cancer cell lines (Dodd et al. 1983; Dokoh et al. 1984; Freake et al. 1984; Pan and Price 1984; Garland et al. 1985; Sher et al. 1985). The synthesis of calbindin D-28K in intestine and kidney is dependent on 1α,25$[OH]_2D_3$ (for review see Wasserman and Fullmer 1983) and its distribution was therefore investigated in various tumour cell lines.

The S-100 proteins (for review see Donato 1986) have been detected in cell lines of human malignant melanomas (Gaynor et al. 1980) and in primary tumours of the nervous system of man (Lauridola et al. 1984; Takahashi et al. 1984) and rat (Haglid et al. 1973).

These findings and the discovery of the tumour-specific Ca^{2+}-binding proteins such as oncomodulin (MacManus and Brewer 1987; Gillen et al. 1987) and the tumour-associated protein (M_r 12K; Pfyffer et al. 1984a) immunologically related to parvalbumin (Heizmann 1984; Heizmann and Celio 1987; Heizmann and Berchtold 1987) prompted us to look for the intracellular distribution of specific Ca^{2+}-binding proteins, using immunohistochemical and biochemical methods.

Materials and methods

The LICR(Lond)-HN6 cell line was derived from a human squamous carcinoma of the tongue (Easty et al. 1981a, b; 1986). The Caco-2 cell line was derived from a human colon

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adenocarcinoma (Pinto et al. 1983; Zweibaum et al. 1983; Grasset et al. 1984; Rousset et al. 1985) and kindly provided by Dr. A. Zweibaum (Unité des Recherches sur le Métabolisme et la Différentiation des Cellules en Culture, Hôpital Broussais, Paris, France). Neuroblastoma cells, clone NB-2a, derived from the C-1300 mouse tumour cell line (Augusti-Tocco and Sato 1969), and glioma cells, clone C-6, derived from a N-nitrosomethylurea-induced rat glial tumour (Benda et al. 1968) were obtained from the American Type Culture Collection (Rockville, MD, USA) and provided by Dr. D. Monard (Friedrich Miescher Institut, Basel, Switzerland).

For immunocytochemical studies cells were grown on glass coverslips. The LICR(Lond)-HN6 cells were cultivated as described (Easty et al. 1981b). Caco-2 cells were routinely grown as monolayers in 75 cm^2 plastic flasks (Corning Glassworks, Corning, NY, USA) in Dulbecco's modified Eagle's minimum essential medium (glucose content; 25 mmoles/l) in a CO_2/air (10/90) atmosphere. The medium was supplemented with 20% fetal bovine serum, 1% non-essential amino acids and 100 I.U. penicillin/ml and 100 µg streptomycin/ml.

The monospecific rabbit and guinea pig antisera against rat muscle parvalbumin have been previously characterized (Pfyffer et al. 1984b). The latter antisera crossreact strongly with human and mouse parvalbumin (Berchtold et al. 1985). The antisera against chicken calbindin D-28K was a gift of Dr. A.W. Norman (Department of Biochemistry, University of California at Riverside, CA, USA). Anti-bovine brain S-100 serum raised in rabbits was obtained from Dakopatts (Santa Barbara, CA, USA) and monoclonal antibody against rat tubulin was purchased from Monosan (Sanbio, Nistelrode, The Netherlands).

For light microscopy cells were immunostained and photographed as described previously (Pfyffer et al. 1984 a, b; Pfyffer et al. 1987). Dilutions of the antisera used were as follows: rabbit anti-parvalbumin and guinea pig anti-parvalbumin (1/30), rabbit anti-calbindin D-28K (1/100), rabbit anti-S-100 (1/50), and rabbit anti-tubulin (1/50). Pre-immune sera were diluted accordingly. Rabbit anti-parvalbumin serum was preabsorbed with antigen as previously described (Heizmann and Celio 1987). Fluorescein isothiocyanate(FITC)-conjugated goat anti-rabbit IgG (1/150), FITC-conjugated goat anti-guinea pig IgG and tetramethyl rhodamine isothiocyanate(TRITC)-conjugated goat anti-rabbit IgG (1/150) were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands) and used at a dilution of 1/150.

For electron microscopy gold grids were washed and sandwiched between 0.5% Formvar and a round glass coverslip (Webster et al. 1978) and sterilized by ultraviolet irradiation. Freshly trypsinized LICR(Lond)-HN6 cells were seeded on the coverslips and kept under culture conditions (Easty et al. 1981b) for 24 h. The cells were then washed with phosphate buffered saline (PBS; BR14A, Oxoid, Basingstoke, GB). For the cytoskeleton preparation, cells were extracted for 5 min with a buffer containing 2 mM EGTA, 2 mM PIPES, 80 mM KCl, 5 mM $MgCl_2$, and 1% Nonidet P-40 (pH 6.9) and washed in an identical buffer without Nonidet. Cells were fixed with 3% formaldehyde in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ and immunostained with either rabbit anti-parvalbumin or preimmune serum 1/30. Subsequently, cells were incubated with colloidal gold-labelled goat anti-rabbit IgG 1/20 (*GARG 10*; Janssen Life Science Products, Beerse, Belgium). The labeled cells were first washed with PBS (also containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$), then stabilized in 3% glutaraldehyde, extensively washed with double-distilled water and cryofixed by immersion into liquid propane (−180° C). The grids were freeze-dried in a Balzers freeze etching apparatus, model BAF 300 (Walther et al. 1984), and coated with carbon by rotary shadowing at an angle of 40°. Cells were taken using AGFA Scientia 23 D 56 cutfilms. To obtain stereopairs the samples were tilted by 6°.

Biochemical analyses were performed as follows: tumour cells were extracted in 2 vol of 4 mM EDTA (pH 7.4) containing various protease inhibitors: leupeptin (1 µM), phenylmethylsulfonyl fluoride (4 mM), pepstatin (1 µM), L-1-(tosylamido)-2-phenylethyl-chloromethyl ketone (15 mM), trypsin inhibitor (0.1 g/ml), Trasylol (30 U/ml). The extracts were subjected to heat treatment as described (Pfyffer et al. 1984a). Purified rat muscle parvalbumin was used as standard for the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and high performance liquid chromatography (HPLC). Protein extracts were labelled in vitro by reductive methylation with [^{14}C] formaldehyde (specific activity: 47 mCi/mmol, 1 Ci = 37 GBq, New England Nuclear) and analyzed on 2D-gels. Extracts were separated by HPLC on reverse phase supports (Aquapore RP-300 cartridge; particle size, 10 µM; pore size, 300 Å; Brownlee Labs., Santa Clara, CA, USA) using a 25 mM Tris-HCl (containing 0.1 mM EGTA)/acetonitril (pH 7.4) buffer system (Pfyffer et al. 1984a).

Results

In well-isolated LICR(Lond)-HN 6 cells or in cells located at the periphery of loose "islets," the cytoplasm exhibited a pronounced staining with anti-parvalbumin serum, whereas the cytosol of cells buried in heavier "islets" remained non-reactive (Fig. 1b; asterisk). The nuclei of most cells were labelled (Fig. 1b; arrow). Micrographs at higher magnification demonstrate that the nuclei (*N*) of mitotic (Fig. 1c) and of some non-dividing cells (Fig. 1d) were particularly strongly labeled, and furthermore, they revealed in some cells, immunoreactivity associated with filamentous perinuclear structures (Fig. 1d). However, a presence of tubulin can probably be excluded since double-labelling experiments revealed two completely different patterns for the two antigens. The staining patterns were also different from actin which stained only filaments at the periphery of the cell (results not shown).

Immune-electron microscopy was applied to localize the parvalbumin-reactivity more precisely (Fig. 2). Under the conditions used, LICR(Lond)-HN6 cells retained their usual shape and motility behaviour, but grew more slowly than cells seeded on the usual glass substrate. After removal of the cytoplasm by extraction, the cytoskeletal structures remained intact. The typical filamentous network is shown in Fig. 2a. In cells incubated with anti-parvalbumin serum, followed by colloidal gold-staining, the gold label (Fig. 2b; arrowheads) was unevenly distributed within the cytoskeleton and clustered at some filaments. Stereopairs (Fig. 2c) allowing a three-dimensional localization of parvalbumin-immunoreactivity confirm the localization of the label at the filaments (Fig. 2c: arrow-

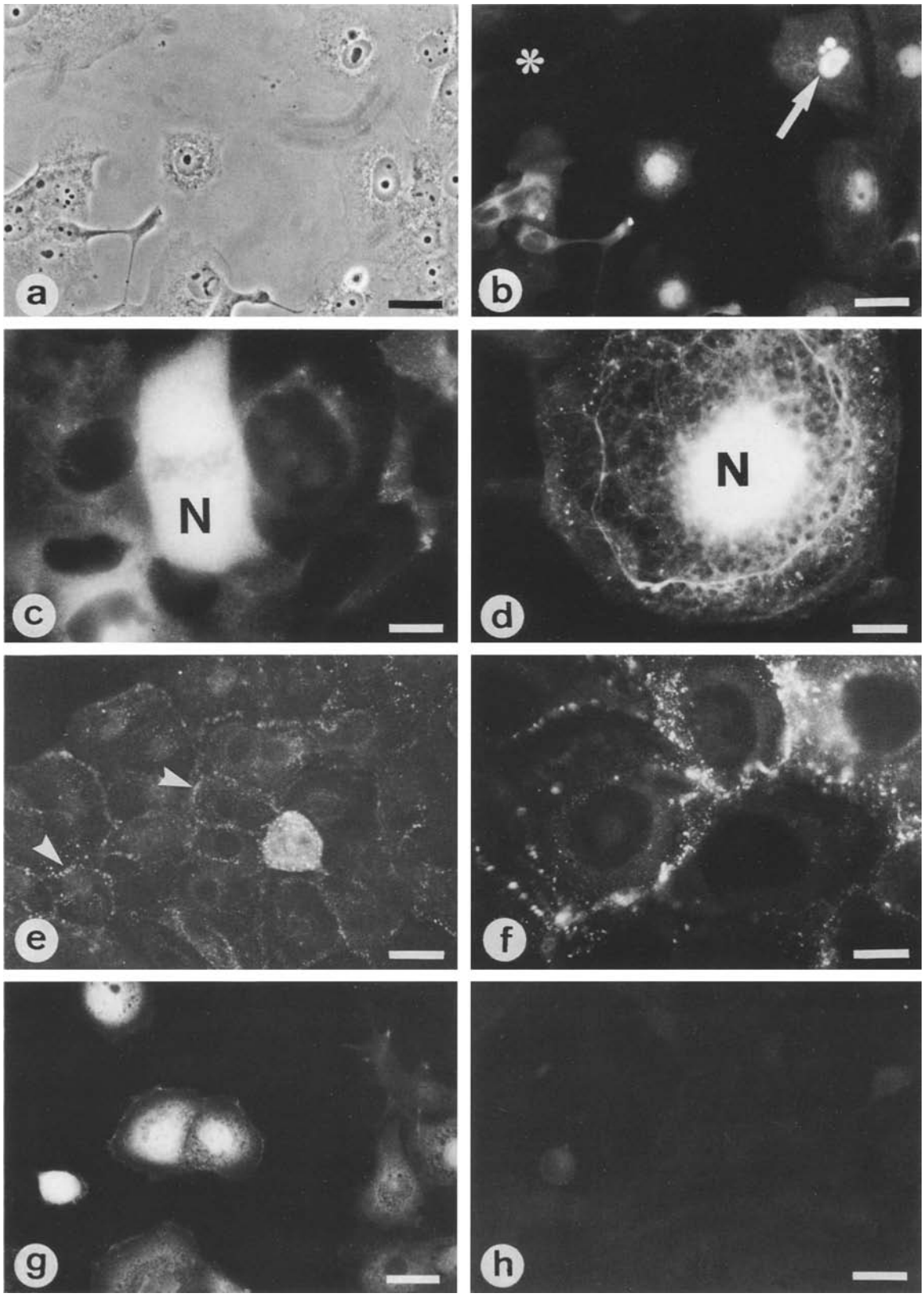

Fig. 1 a–b. Immunocytochemistry of the human LICR(Lond)-HN6 carcinoma cell line with antisera to Ca^{2+}-binding proteins. (**a**) phase contrast micrograph corresponding to (**b**); (**b–d**) indirect immunofluorescence with rabbit anti-rat parvalbumin serum; (**e, f**) rabbit anti-calbindin D-28K serum; (**g**) rabbit anti-S-100 serum; (**h**) rabbit pre-immune serum (control). *Asterisk* denotes an unlabeled cell, *arrow* denotes a labeled nucleus, *arrowheads* point to the membraneous staining; *N*, nucleus. Magnifications: (**a, b, e, g, h**) × 500, (**c, d, f**) × 1000; *bar*: (**a, b, e, g, h**) = 20 μm, (**c, d, f**) = 10 μm

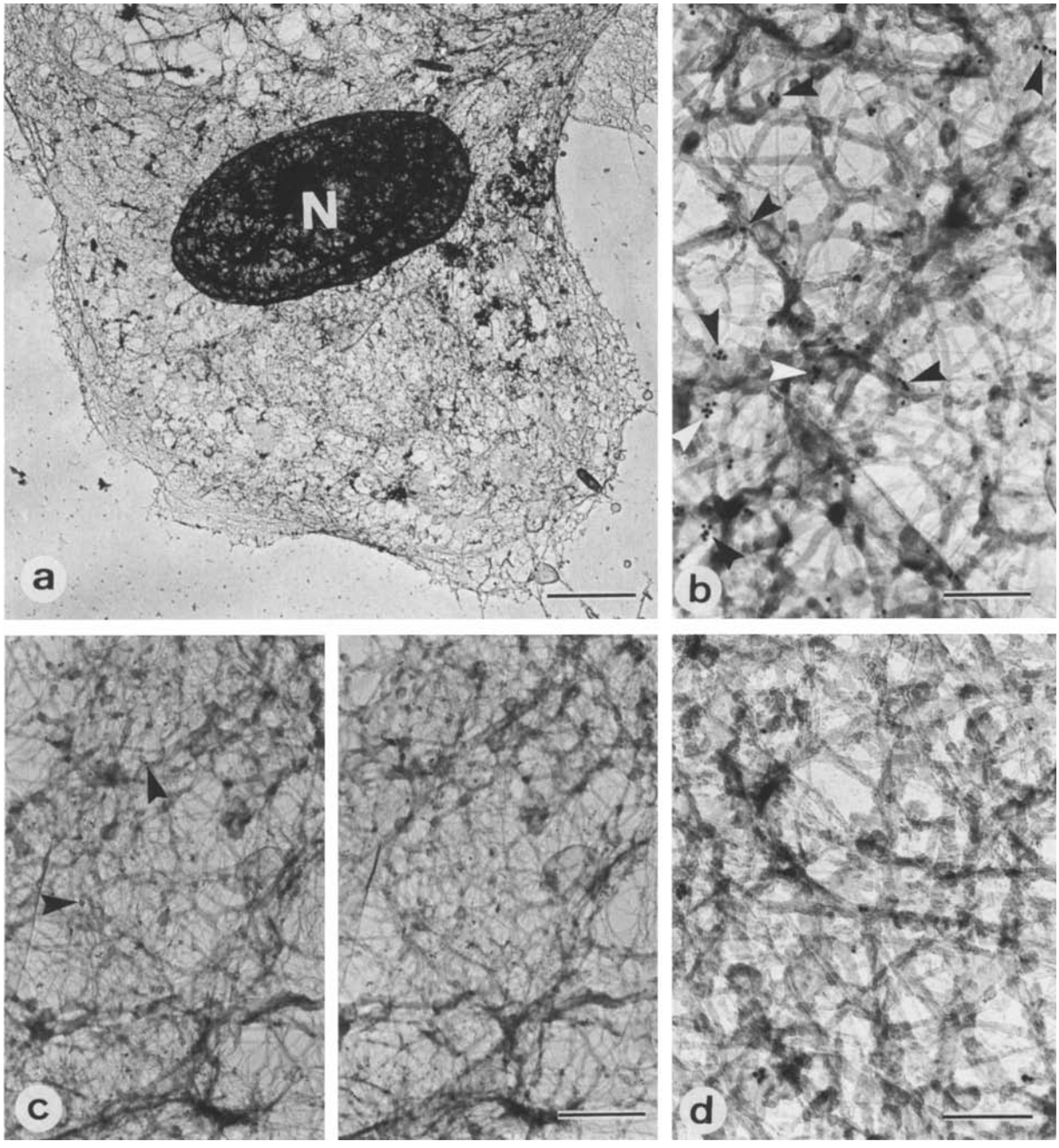

Fig. 2a–d. Electron microscopy of human LICR(Lond)-HN6 carcinoma cell line. **(a)** Electron micrograph at low magnification of an extracted cell immunostained with rabbit anti-parvalbumin serum labeled with colloidal gold. The cell shows a typical filamentous network. **(b)** electron micrograph of the cytoskeleton of the same cell at higher magnification showing the distribution of the gold particles (*arrowheads*) associated to filaments. **(c)** stereo-micrograph of the same cell revealing an alignment of the gold particles to the filaments (*arrowheads*). **(d)** LICR(Lond)-HN6 cell treated with pre-immune serum and colloidal gold (control). Magnifications: **(a)** × 3000, **(b, d)** × 60000, **(c)** × 30000; *bar*: **(a)** = 5 μm, **(b, d)** = 250 μm, **(c)** = 500 μm; *N*, nucleus

heads). Cells treated with the pre-immune serum (control: Fig. 2d) or incubated with the second antibody only were negative (not shown).

A distribution different to that observed for anti-parvalbumin was found when LICR(Lond)-HN6 cells were incubated with an antiserum against calbindin D-28K. Generally, staining was found at the cell membranes (Fig. 1e, f: arrowheads) but not in the cytoplasm or nuclei; occasionally, the entire cell was faintly labelled. S-100

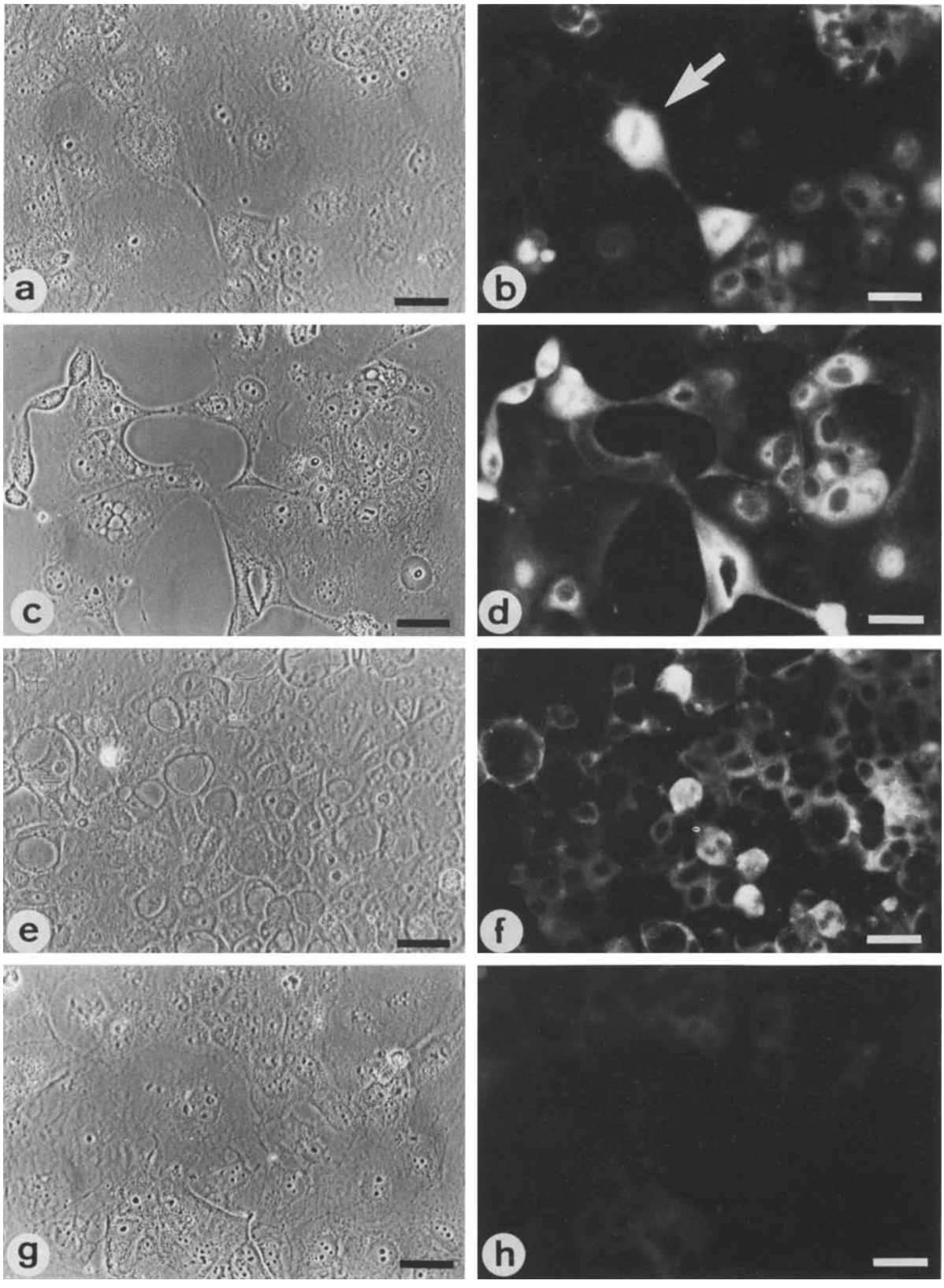

Fig. 3a–h. Indirect immunofluorescence of parvalbumin and calbindin D-28K in 3-day-old cultures of Caco-2 cells. Phase-contrast micrographs (**a, c, e, g**) corresponding to indirect immunofluorescence (**b, d, f, h**) with rabbit anti-parvalbumin serum (**b, d**), rabbit anti-calbindin D-28K serum (**f**), rabbit pre-immune serum (**h**: control). *Arrow* points to a mitotic cell. Mangification: (**a–h**) × 250; *bar* (**a–h**) = 40 μm

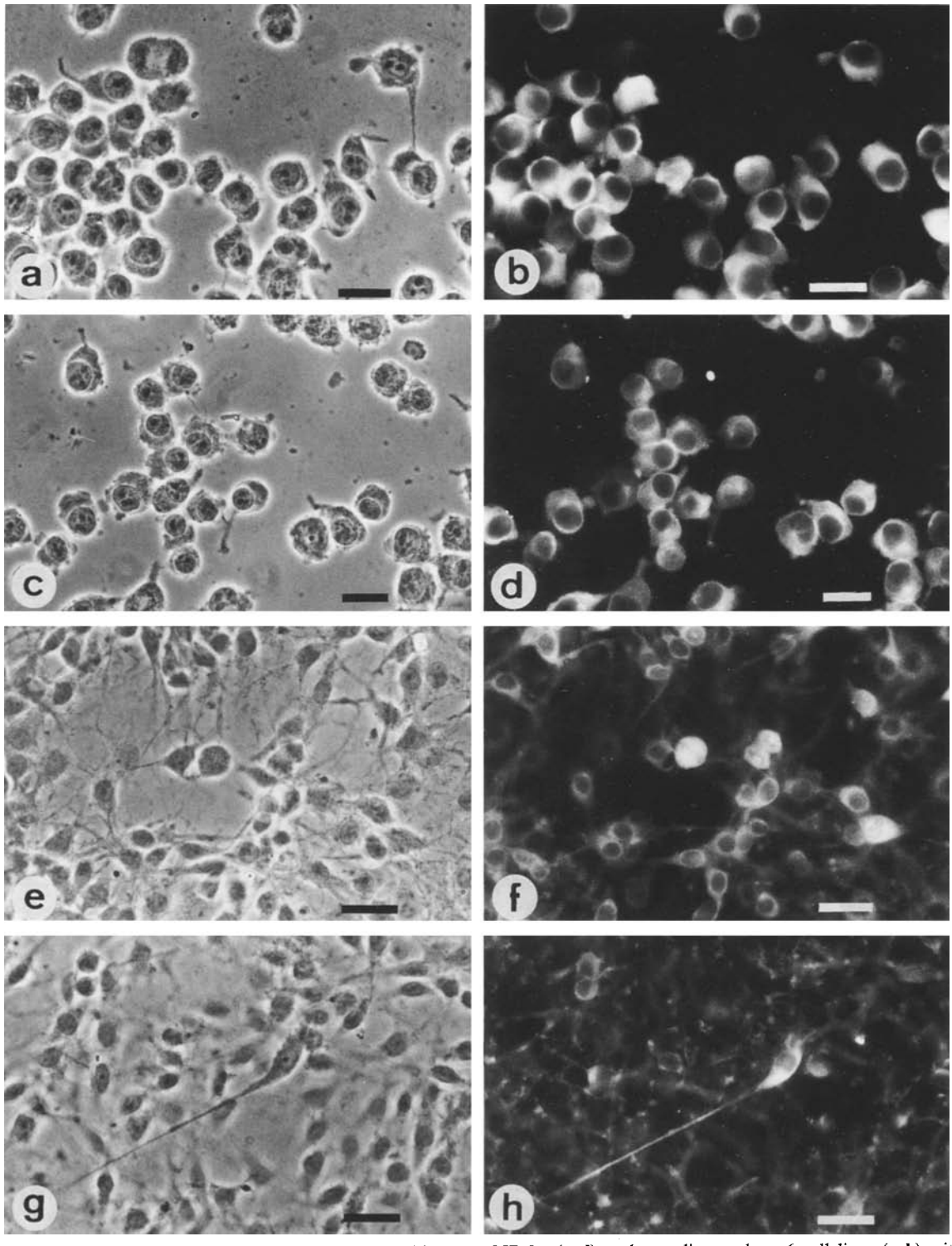

Fig. 4a–h. Immunocytochemistry of mouse neuroblastoma NB-2a (**a–d**) and rat glioma clone-6 cell lines (**e–h**) with antisera to parvalbumin and S-100 proteins. Phase-contrast micrographs (**a, c, e, g**) corresponding to indirect immunofluorescence (**b, d, f, h**) with rabbit anti-parvalbumin serum (**b, f**), rabbit anti-S-100 protein serum (**d, h**). Magnifications: (**a, b, e, f, g, h**) × 470, (**c, d**) × 417; *bar*: (**a–h**) = 20 μm

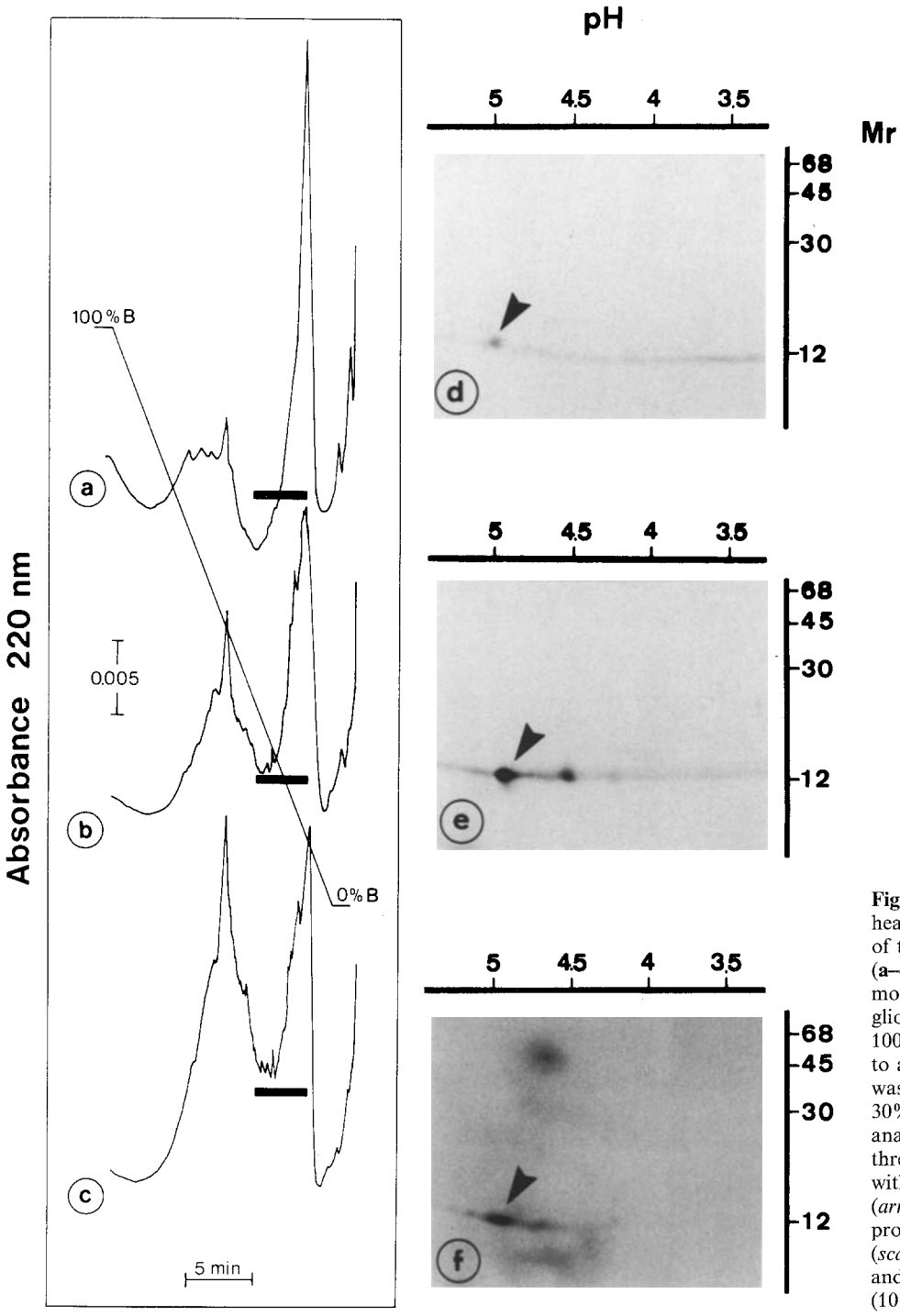

Fig. 5a–f. Biochemical analysis of heat-treated 4 mM EDTA extracts of three tumour cell lines. HPLC (**a–c**) of (**a**) human Caco-2, (**b**) mouse neuroblastoma and (**c**) rat glioma cell line. In each run 80–100 µg of protein (corresponding to approximately 9×10^6 cells) was loaded. Proteins eluting at 7–30% buffer B (*bar*), were further analyzed by 2D-PAGE (**d–f**). All three extracts contain a protein with $M_r = 12K$ and pI = 4.8 (*arrowhead*). [^{14}C]-labelled proteins were identified by pI (*scale* on top) and M_r ($\times 10^{-3}$) and visualized by fluorography (10 days exposure)

immunoreactivity in LICR(Lond)-HN6 cells was concentrated in the cytoplasm and nucleus (Fig. 1g). Cells incubated with the appropriate pre-immune sera (Fig. 1h) did not stain.

The Caco-2 cell line exhibits a typical enterocytic differentiation spontaneously (Pinto et al. 1983; Zweibaum et al. 1983; Grasset et al. 1984; Rousset et al. 1985). After 3 days in culture, Caco-2 cells form subconfluent monolayers, comprised of well-delimited, polygonal cells (Fig. 3a, c, e, g). After treatment with anti-parvalbumin serum, the majority of the cells showed a homogeneous staining of the cytoplasm, leaving the nucleus unlabeled (Fig. 3b, d). Mitotic cells (Fig. 3b: arrowhead) and

cells at the edge of clusters exhibited the most intense labelling (Fig. 3d). Only background staining was observed with cells treated with the corresponding pre-immune serum (Fig. 3h).

When Caco-2 cells were exposed to anti-calbindin D-28K serum, the membranes of most and the cytoplasm of a few cells were faintly labelled (Fig. 3f). Overall distribution of calbindin D-28K immunoreactivity in Caco-2 cells (Fig. 3f) resembled the distribution found in the LICR(Lond)-HN6 line (Fig. 1e, f).

Figure 4a–d summarizes the labeling with antisera against parvalbumin, calbindin D-28K, and S-100 proteins in a mouse neuroblastoma cell line. All cells gave a positive immunoreaction with anti-parvalbumin serum, i.e. a reaction which was evenly distributed in the cytoplasm; in contrast, nuclei remained unlabelled (Fig. 4b). Treatment of the neuroblastoma cells with the anti-S-100 serum developed a strong cytoplasmic staining (Fig. 4d); in contrast, the fluorescence did not exceed background level when cells were incubated with anti-calbindin D-28K serum (results not shown).

The cytoplasm of most rat glioma cells stained with anti-parvalbumin serum at various intensities (Fig. 4f). Anti-S-100 staining was found mainly in large cells with long processes (Fig. 4h); no immunoreactivity was observed for the anti-calbindin D-28K serum (not shown).

In biochemical analyses the extracts of the tumour cell lines were analyzed by HPLC (Fig. 5a–c) and 2D-PAGE (Fig. 5d–f) for the presence of the Ca^{2+}-binding proteins. After having observed a pronounced immunoreactivity of the cell lines with antisera against parvalbumin, we focussed mainly on the presence of this protein. However, in none of the HPLC analyses of the different cell lines was parvalbumin found, but all the extracts showed the presence of a protein eluting at 22–24.5% B. The protein eluted in this peak was pooled and lyophilized after dialysis and could be analyzed conveniently on 2D-PAGE thereafter. In all the cell lines tested, parvalbumin was absent thus confirming the results of HPLC-analysis. Rather, a protein with M_r of 12K and pI of 4.8 was be found which has been discovered in a previous study (Pfyffer et al. 1984a) and presents a tumour-associated protein which crossreacts with the anti-parvalbumin serum.

Discussion

In our study, we have observed a pronounced cytosolic immunoreactivity against an anti-parvalbumin serum in all four cell lines. In some cells of the human carcinoma cell line LICR(Lond)-HN6, both light and electron microscopy indicated, in addition, a positive immunoreaction associated with some filaments which are distinct from tubulin and actin structures. The immunoreactivity persisted even after a harsh treatment of the cells with a detergent-containing buffer, a necessary step prior to electron microscopy.

The results of the biochemical analyses (HPLC: Fig. 5a–c/2D-PAGE: Fig. 5d–f) demonstrate that parvalbumin itself is absent in all these cell lines, but instead, a protein with M_r 12K, pI 4.8 and eluting at 22–24.5% B was observed. These properties coincide perfectly with those of the tumour-associated protein which has previously been found to crossreact with anti-parvalbumin serum (Pfyffer et al. 1984a). The persistent presence of this tumour-associated protein (M_r 12K) in all the tumour cell lines tested so far leads to the suggestion that it may – like the tumour-specific oncomodulin (MacManus and Brewer 1987) – help in decreasing the elevated concentrations of Ca^{2+} and therefore, modulate the enhanced stimulation of Ca^{2+}-dependent processes such as motility and cell proliferation.

Among the cell lines reacting with an anti-S-100 serum, the glioma cells, particularly those with long processes, exhibited a strong staining. This is not suprising since, in addition to certain cell lines (Ishikawa et al. 1983), glioma cells are known to contain and secrete S-100 proteins (Benda et al. 1968).

Finally, the intracellular distribution of the S-100 proteins and of the tumour-associated protein (M_r 12K) differs from the cellular localization of calbindin D-28K in two ways: (1) the latter protein is clearly absent from neuroblastoma and glioma cells and (2) its immunoreactivity is restricted almost exclusively to the cell membrane. Since $1\alpha,25(OH)_2D_3$-receptors have been found on membranes also of human breast tumours and tumour cell lines (Freake et al. 1984; Garland et al. 1985; Sher et al. 1985), it is likely that the synthesis of this protein in tumour cells may be dependent on vitamin D as it is in intestine and kidney (Wasserman and Fullmer 1983).

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