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Original Paper

Neuroblastoma Cells Can Actively Eliminate Supernumerary *MYCN* Gene Copies by Micronucleus Formation—sign of Tumour Cell Revertance?

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Human neuroblastoma cell lines frequently exhibit *MYCN* amplification and many are characterised by the presence of morphologically distinct cell types. The neuronal cells (N-cells) and the so-called flat cells (F-cells) are thought to represent manifestations of different neural crest cell lineages and are considered to be the consequence of neuroblastoma cell pluripotency. In this study, various neuroblastoma cell lines were examined for micronuclei. In F-cells of neuroblastoma cell lines with extrachromosomally amplified *MYCN*, we observed the frequent occurrence of micronuclei. Using fluorescence *in situ* hybridisation (FISH) with a *MYCN* specific probe, we demonstrated that these micronuclei were packed with *MYCN* hybridisation signals. In addition, in a minor percentage of cells, *MYCN* signals occurred in clusters, adhered to the nuclear membrane and aggregated in nuclear protrusions. In F-cells, a substantial reduction or lack of amplified *MYCN* copies was observed. These observations let us conclude that extrachromosomally amplified genes can be actively eliminated from the nucleus resulting in a dramatic loss of amplified sequences in the F-cells. Moreover, reduction or loss of amplified sequences in F-cells was shown to be accompanied by downregulation of *MYCN* expression, by a decrease in proliferative activity and by upregulation of molecules of the major histocompatibility complex class I (MHC I). Interestingly, F-cells are not restricted to neuroblastoma cell cultures, but also occur in cell lines of other tissue origin. All F-cells share important biological features, interpreted as cell revertance, i.e. loss of the malignant phenotype and properties. This fact, together with the demonstration that neuroblastoma cells do not differentiate into Schwann cells *in vivo* [1] Ambros et al. *NEJM* 1996, 334, 1505–1511, do not support the hypothesis that F-cells represent Schwannian/glial differentiation *in vitro*. We therefore postulate that the elimination of amplified *MYCN* gene copies in cultivated neuroblastoma cells is in line with the phenomenon of tumour cell revertance. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, cell lines, N-cells, F-cells, micronuclei, *in situ* hybridisation, *MYCN* amplification, *MYCN* expression, proliferative activity, MHC I, revertant cells
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INTRODUCTION

AMPLIFICATION OF oncogenes has been observed in a wide variety of human tumours and is known to be associated with a poor prognosis. In neuroblastoma, an embryonal tumour of the sympathetic nervous system, amplification of the proto-oncogene *MYCN* is described in up to 20% of primary tumours associated with poor prognosis irrespective of age and clinical stage [2, 3]. The *MYCN* gene is a developmentally regulated proto-oncogene (for review see [4]), structurally related to c-*myc* and was originally described as being amplified in neuroblastoma cell lines [5]. The gene maps to the chromosomal region 2p23-24 [6] and encodes for a DNA binding protein [7]. Cytogenetic manifestations of amplified genes are acentric extrachromosomal elements, the double minute chromosomes (dmin) and homogeneously staining regions (hsr) which are integrated at different chromosomal sites.

Cell lines established from human neuroblastomas usually exhibit *MYCN* amplification in dmin, more frequently than hsr. Furthermore, most neuroblastoma cell lines are

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characterised by the presence of morphologically distinct cell types [8–10]. The so-called N-cells exhibit a neuronal phenotype with relatively small nuclei, scant cytoplasm and neurite-like cell processes. The other cell type lacks neuronal features [11–13]. These cells are known as flat cells (F-cells) or substrate adherent cells (S-cells) with marked enlargement of nuclei and cytoplasm. A third cell type displays intermediate (I-cells) morphology, most likely representing a transitional state between N- and F-cells. Treatment of neuroblastoma cell lines with differentiating agents can augment neurite outgrowth in N-cells or raise the number of F-cells [13–17]. Previously, various cell types have been considered to correspond to the F-cell phenotype: glial or Schwann cells [12, 13, 16, 18], melanocytes [11, 13], fibroblast-like meningeal cells [19] and multipotent embryonal precursor cells of the neural crest [20]. Here, we describe frequent generation of micronuclei in neuroblastoma cell lines, especially occurring in F-cells. Using FISH, we characterised sequences entrapped into micronuclei and determined number and localisation of the amplified *MYC*N copies in the different cell types of three dmin containing neuroblastoma cell lines and two neuroblastoma cell lines with NMA organised in hsr. The proliferative activity of the different cell types was assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation. N-*myc* expression and expression pattern of several neural and non-neural antigens, including MHC class I molecules, was determined by immunocytochemistry.

MATERIALS AND METHODS

Neuroblastoma cell lines

Eleven cell lines, STA-NB-1–STA-NB-11, were established in the Children's Cancer Research Institute (CCRI) in Vienna. The cell line LA-N-1 was obtained from the American Type Culture Collection (ATCC) and FR was kindly provided by Dr Hernaiz-Driever (Frankfurt, Germany). Cells were grown over glass coverslips and fixed with paraformaldehyde or chilled acetone.

Southern blot

Southern blotting using the probe *pNb1* for *MYC*N (kindly provided by Dr Schwab, Heidelberg, Germany) was performed according to standard protocols [21].

Fluorescence in situ *hybridisation (FISH)*

Double-target FISH was performed according to protocols described previously [22, 23]. Neuroblastoma cells were fixed in 4% paraformaldehyde. Predigestion of the cells was carried out in 0.005% pepsin in 0.01N HCl for 3–7 min. For detection of deletion events, we used the probes *D1Z1* (*pUC1.77*) (kindly provided by Dr T. Cremer, Heidelberg, Germany) hybridising to the centromere of chromosome 1 and *D1Z2* (*p1-79*) (obtained from the ATCC), specific for the chromosomal region 1p36.33. The *MYC*N gene was detected with the cosmid probe *pNb101* or the plasmid probe *pNb9* (both kindly provided by Dr Schwab, Heidelberg, Germany). The chromosome 2 centromere-specific probe *D2Z* (Oncor Inc., Gaithersburg, Maryland, U.S.A.) was used as internal control. Denaturation was performed at 80–85°C. Biotin-labelled probes were detected by using a mouse-anti-biotin antibody and a rhodamine-conjugated rabbit-anti-mouse antibody (1:20 and 1:30, DAKO, Glostrup, Denmark). Digoxygenin-labelled probes were detected with a fluoresceine-conjugated sheep-anti-digoxygenin antibody (1:100, Sigma, St. Louis, Missouri, U.S.A.) and a fluorescein-conjugated rabbit-anti-sheep antibody (1:100, DAKO, Glostrup, Denmark). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole-2HCl (DAPI).

MYC*N expression*

*MYC*N expression was determined using the monoclonal *MYC*N antibody B8.4.B. (kindly provided by Dr A. Wenzel, Heidelberg, Germany [24]). The neuroblastoma cells were fixed in chilled acetone (−20°C) for 20 min. The antibody was detected with a rhodamine-conjugated goat-anti-mouse antibody (1:100, Dianova, Hamburg, Germany).

BrdU incorporation

Cells were exposed to BrdU (5–10 μg/ml culture medium) for 120 min and then fixed with 4% paraformaldehyde. Incorporated BrdU was detected with a mouse anti-BrdU antibody (1:100, Boehringer Mannheim Biochemica, Germany) and a rhodamine-conjugated goat-anti-mouse antibody (1:100, Dianova, Hamburg, Germany) following denaturation at 80–85°C.

Immunocytochemistry

The following antibodies were used: anti-neurofilament 160kd, anti-vimentin (both Boehringer Mannheim Biochemica, Germany), anti-Nb84a, anti-chromogranin, anti-S-100, anti-cytokeratin, anti-HLA-ABC (all DAKO, Glostrup, Denmark), anti-CD44 and anti-HLA-DR (both Immunotech, Marseille, France). All antibodies were monoclonal with the exception of anti-Chromogranin and anti-S100 recognising both α and β chains of the protein. Paraformaldehyde was used as the fixative for all antibodies excluding anti-HLA-ABC for which cells were fixed with chilled acetone. For anti-S-100, both fixatives were used. Detection of primary antibodies was carried out with fluorescein- and rhodamine-conjugated antibodies according to standard protocols.

RESULTS

Genetic data and cell types observed in neuroblastoma cell lines

Eleven neuroblastoma cell lines were established in the CCRI (STA-NB-1– -11). All showed a deletion at 1p36.3 and 9 out of 11 were also *MYC*N amplified. Seven cell lines were diploid, two triploid and another two tetraploid. Two neuroblastoma cell lines not established in the CCRI (LA-N-1 and FR) with amplified *MYC*N copies organised in hsr, also showed 1p deletions and had a tetraploid and a triploid DNA content, respectively. Genetic data and presence of F-cells in the cell lines investigated are summarised in Table 1. All but one cell line containing dmin were similar in their phenotype and characterised by the coexistence of N- and F-cells (Figure 1a). The percentages of F-cells ranged from 0.1 to 30%. Micronuclei were observed in all cell types in the lines STA-NB-9, -10, and -11 ranging from 1 to 16% (mean 9.1%, 8.7% and 7.6%, respectively, per 1000 nuclei N- and F-cells evaluated) with the highest percentages found in F-cells. F-cells also contained DAPI positive autofluorescent bodies in the cytoplasm, which possibly represent micronuclei with degraded DNA. The cell line STA-NB-1.2 differed morphologically from the other cell lines with dmin. The cells did not show development of unequivocally identifiable neuritic processes and the occurrence of F-cells was not observed. Cytogenetically, this cell line presented two types of dmin, different in size. Only the small-sized dmin

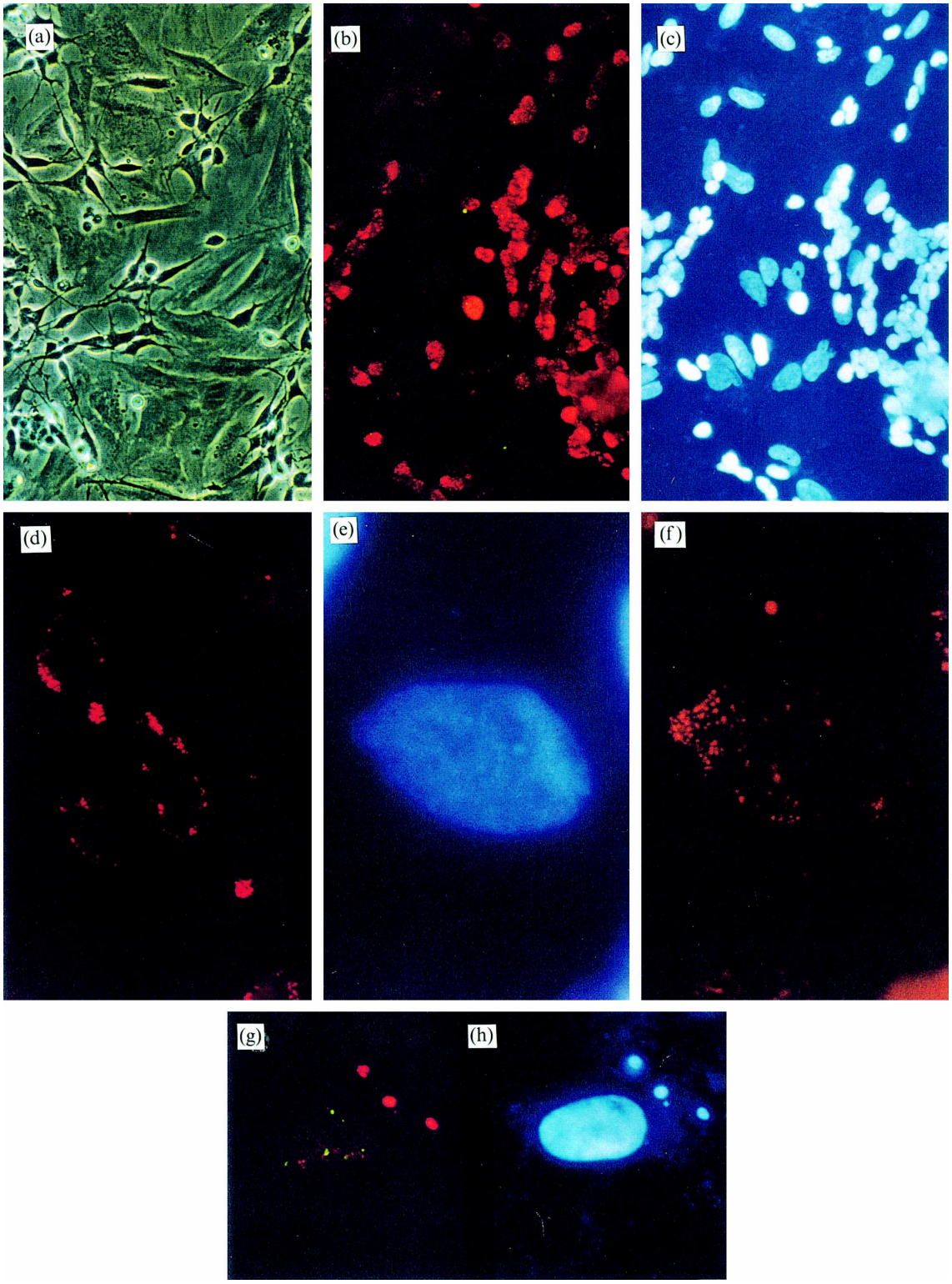

Figure 1. (a) The neuroblastoma cell line STA-NB-10, displaying morphologically different cell types. The smaller N-cells show scant cytoplasm and neuritic processes, the F-cells, in contrast, display pronounced nuclear and cytoplasmic enlargement and, morphologically, lack neuronal features. (b) FISH performed with the *MYCN* probe pNb9 (red). Multiple copies are seen in the smaller round nuclei, whereas the bigger oval shaped nuclei contain only few hybridisation signals or even lack signs of *MYCN* amplification. (c) The same microscopical field as in (b), given a nuclear counterstain with DAPI. Small nuclei correspond to N-cells, the large nuclei to F-cells (same magnification). (d) Enlarged oval nuclei with clusters of *MYCN* signals sticking to the nuclear membrane. In addition, two micronuclei hybridising to the pNb9 probe can be seen. (e,f) In this nucleus, *MYCN* hybridisation signals are concentrated in one region that can be interpreted as a nuclear protrusion; in (e), hybridisation signals and nuclear stain r (DAPI) are highlighted simultaneously, in (f), only the red fluorescence of the *MYCN* hybridisation signals are shown. (g) F-cell nucleus lacking *MYCN* amplification. This nucleus contains four centromeres of chromosomes 2 (labelled in green) with single copies of the *MYCN* gene and three micronuclei heavily labelled with the *MYCN* probe. (h), the same cell as in (g) is shown, stained with DAPI.

Table 1. *Genetic data and presence of F-cells in* 13 *neuroblastoma cell lines*

Cell line	del 1p36.33	*MYCN* amplification	Ploidy*	F-cells
STA-NB-1.2	+	dmin	2n	–
STA-NB-2	+	—	2n	–
STA-NB-3	+	dmin	3n	+
STA-NB-4	+	dmin	4n	+
STA-NB-5	+	dmin	2n	+
STA-NB-6	+	—	3n	–
STA-NB-7	+	dmin	4n	+
STA-NB-8	+	dmin	2n	+
STA-NB-9	+	dmin	2n	+
STA-NB-10	+	dmin	2n	+
STA-NB-11	+	dmin	2n	+
LA-N-1	+	hsr	4n	–†
FR	+	hsr	3n	–

*2n, 3n, 4n denotes a near-diploid, near-triploid and near-tetraploid DNA content in most cell lines.
†See discussion.

hybridised to the *MYCN* probe. The other, larger dmin most likely represented amplification of another chromosomal region. The cell line STA-NB-6, lacking *MYCN* amplification, was composed of a relatively uniform cell type with pronounced neuronal features. Rarely, enlarged cells, not resembling the F-cells in the other cell lines, were found. In the cell lines with hsr (LA-N-1 and FR) we observed giant cells, often multinucleated, but not the occurrence of typical F-cells.

Reduction or lack of MYC*N gene amplification in flat cells—micronuclei contain amplified* MYC*N genes*

The *MYCN* gene copy number and distribution within the N- and F-cells of three cell lines with dmin (STA-NB-9, -10, -11) were characterised in more detail. Besides a marked heterogeneity in *MYCN* copy number within individual cells of a cell line, ranging from single copies to more than a hundred copies (Figure 1b and c), we observed a peculiar distribution of the *MYCN* genes within a small proportion of nuclei. Firstly, the majority of hybridisation signals within a single nucleus clustered and were stacked close to the nuclear membrane (Figure 1d); secondly, nuclear protrusions were seen which were filled with copies of the amplified gene (Figure 1e and f); thirdly, most of the micronuclei observed showed hybridisation signals with the *MYCN* probe *pNb9* (Figures 1g and h). Usually, N-cells with *MYCN* positive micronuclei also contained supernumerary copies within the nucleus (not shown). In contrast, F-cells showed a dramatic reduction of the *MYCN* copy number or even lacked gene amplification (Figures 1g and h). Micronuclei were also observed in the LA-N-1 and FR cell lines with amplified gene copies organised in hsr, but rarely hybridised to the *MYCN* probe (<1%). Numbers of *MYCN* copies in N- and F-cells in the three cell lines with dmin are given in Figure 2 (500 N-cell nuclei and 300 F-cell nuclei were evaluated per slide).

MYC*N expression in N- versus F-cells*

To determine whether the reduction of *MYCN* gene copies is accompanied by a reduction in *MYCN* expression, we investigated the expression level in the different cell types using an antibody directed against an N-terminal epitope of the *MYCN* protein [24]. Ninety-five per cent of the N-cells showed detectable expression levels ranging from low to high (Figure 3a and b). Most of the F-cells (88–98%), in contrast, showed no reaction with the antibody used (300 N- and F-cell nuclei evaluated per slide).

BrdU incorporation in N- versus F-cells

BrdU uptake over 120 min was assessed in order to determine whether the loss of amplified sequences and the down-regulation of *MYCN* gene expression in F-cells is accompanied by a decrease in proliferative activity. N-cells greatly differed from F-cells in the number of labelled nuclei (Figures 3c and 4). The incorporation of BrdU was determined in 500 N-cells and at least 200 F-cells per slide.

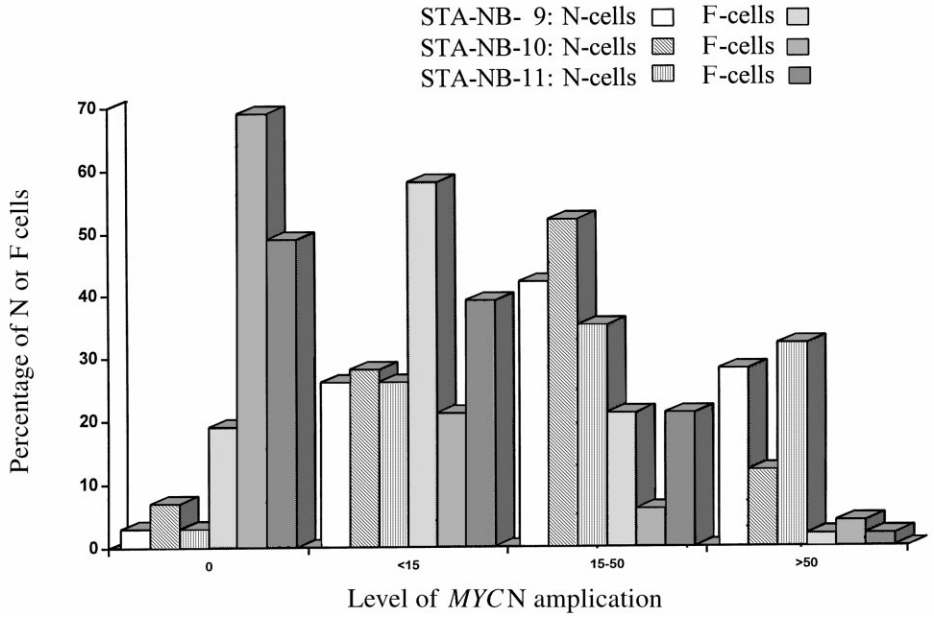

Figure 2. ***MYCN*** **copy number in N- versus F-cells in the cell lines STA-NB-9, STA-NB-10, and STA-NB-11.**

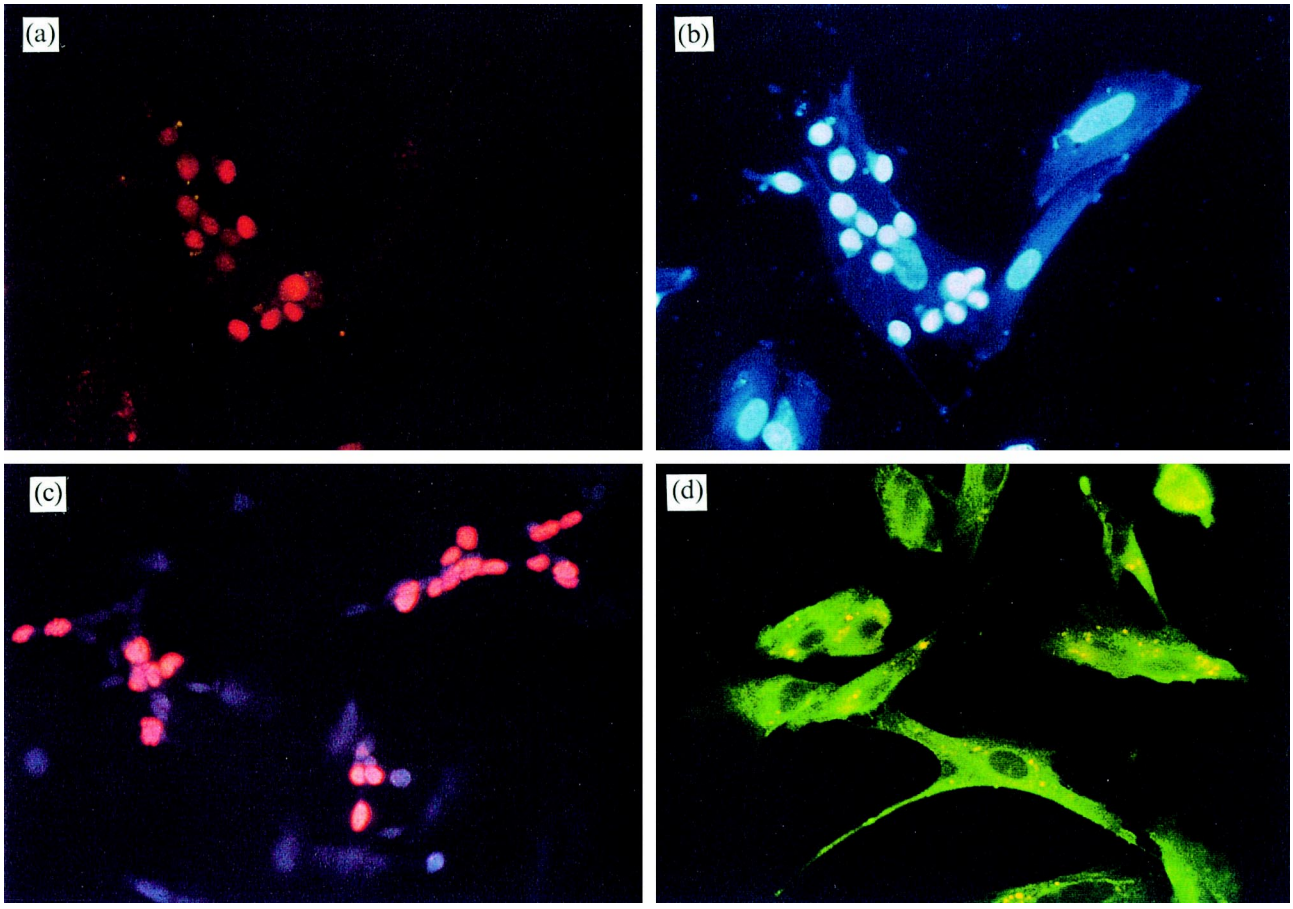

Figure 3. (a) Small round cell nuclei showing weak to strong expression of *MYCN* identified by the antibody B8.4.B. The five F-cell nuclei in this area do not show a reaction with this antibody. (b) Same nuclei as shown in (a), counterstained with DAPI. (c) BrdU uptake identified by an anti-BrdU antibody. Rhodamine labelled nuclei indicate incorporation of the thymidine analogue over 120 min. Many of the nuclei corresponding to N-cells are positive. In contrast, F-cell nuclei shown in this microscopical field do not show evidence of BrdU uptake. (d) HLA-ABC expression identified immunologically. Only cells with abundant cytoplasm (F-cells) are stained. The small N-cells did not react with this antibody.

Immunophenotype of N- and F-cells

The expression pattern of neural and non-neural antigens was analysed in N- and F-cells of the cell lines STA-NB-9, -10, and -11 to characterise the phenotype of these cell types. Neuronal and neuroendocrine markers such as NF 160 kDa, Nb84a and CGA (only a minor percentage of N-cells) were expressed in N-cells in varying intensities. A positive reaction in the majority of N-cells to vimentin was also observed. None of the cells reacted with the anti-cytokeratin antibody. S-100 positivity was dependent on the fixative used. Paraformaldehyde-fixed cells failed to react with the S-100 antibody, whilst acetone fixation revealed a weak staining in N- as well as F-cells. Strong reactions were observed in F-cells with the antibodies to vimentin, CD44 and HLA-ABC (Figure 3d). HLA-DR was negative in both cell types.

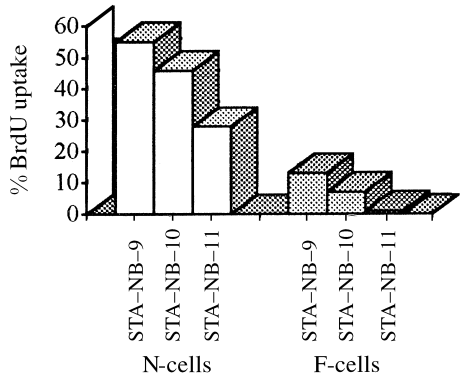

Figure 4. BrdU uptake in N- and F-cells over 120 min in the cell lines STA-NB-9, STA-NB-10, and STA-NB-11.

DISCUSSION

Micronuclei occur spontaneously in cultivated neoplastic and normal cells [25–27], or can be increased in number by chemical agents or by radiation [25, 28, 29]. They have been reported to contain whole chromosomes, e.g. the X and Y chromosomes [27], as well as acentric chromosomes [28, 29]. Loss of amplified gene copies by micronucleus formation has already been described by Von Hoff and associates for the c-*myc* gene following exposure of colon carcinoma cells containing dmin to hydroxyurea [30]. The present study shows that elimination of supernumerary *MYCN* gene copies occurs spontaneously in neuroblastoma cell lines by micronucleation. The peculiar distribution of *MYCN* signals in the interphase nuclei of neuroblastoma cells (see Results and Figure 1d–f) supports the idea of an actively regulated

process, although the underlying mechanism accounting for the selective entrapment of amplified genes is unknown. This process can finally result in a complete loss of supernumerary copies, leading to the formation of F-cells. This is in contrast to the passive unequal distribution of dmin which occurs during cell division. Whether the decrease in *MYC*N expression is a consequence of the loss of amplified sequences or precedes the elimination of *MYC*N copies is not yet clear. In addition, MHC class I molecules, thought to be involved in immunorecognition processes, are upregulated in F-cells. Neuroblastoma tumours infrequently express MHC I, apparently dependent on the level of *MYC*N expression [34]. Interestingly, it has been shown that expression of MHC I molecules is upregulated when *MYC*N gene expression is suppressed in hybrid cell lines [32]. Efficient elimination of amplified genes does not seem to work in cells with hsr as the two hsr-bearing neuroblastoma cell lines rarely contained micronuclei positive for *MYC*N gene hybridisation signals. These results are in agreement with the data published by Von Hoff and associates [30], who observed inducible loss of amplified oncogenes to be restricted to a cell line with dmin, not occurring in a cell line bearing hsr. In addition, we did not observe typical F-cells in two neuroblastoma cell lines with hsr, although we are aware that an F-cell variant of the cell line LA-N-1 was described [18, 33]. In another hsr bearing neuroblastoma cell line, a decrease in *MYC*N expression but no change in the amplification rate has been shown in F-cells [34].

The occurrence of F-cells in neuroblastoma cell cultures is commonly interpreted as a differentiation process and it is concluded that neuroblastoma cells have the capacity to differentiate along divergent cell lineages recapitulating neural crest development [12, 13, 16]. These studies resulted in the view that neuroblastoma may originate from a pluripotent (at least bipotent) neural crest cell and most authors have hypothesised that F- or S-cells represent differentiating or mature glial/Schwann cells. This hypothesis is mainly based on the expression of S-100 β protein, myelin basic protein (MBP) and the enzyme cyclic nucleotidyl phosphodiesterase (CNP) in F-cells. However, S-100 β which is specific for the Schwannian and oligodendrocytic lineage, has also been shown to be present in neuronal cells but masked by aldehyde fixation [35]. MBP has been shown to be expressed not only in myelin producing cells but also in the immune system [36] and CNP activity has been detected in N-cells [18]. In addition, two major observations do not support the hypothesis of Schwannian differentiation *in vitro*: (i) *in vivo*, neuroblastoma cells do not differentiate into Schwann cells [1] and (ii) the occurrence of F-cells seems to be a common phenomenon, not restricted to neuroblastoma cell lines. F-cells have been described to occur spontaneously or chemically induced in cell lines established from, for example, adenocarcinoma of the lung [37], fibrosarcoma [38], pheochromocytoma [39], Ewing's tumour [40] and rhabdomyosarcomas (C.M.H., data not shown) and in cell lines derived from virus-transformed and oncogene-transfected cells [41–44]. Moreover, F-cells of different tissue origin, including F-cells occurring in neuroblastoma cell lines [19, 20, 33], share common properties: a decrease in proliferative activity, contact inhibition, reduction or lack of cloning efficiency in soft agar and reduction or lack of tumorigenicity in nude mice [37–45]. This loss of malignant features is commonly designated as cell revertance. Together, the observations made on F-cells of different tissue origin suggest the presence of a general mechanism not restricted to neuroblastoma cell lines and not necessarily reflecting a differentiation process along a specific lineage.

Based on these facts, we hypothesise that the F-cells in most neuroblastoma cell lines represent a cell type that has lost malignant properties, i.e. a revertant cell. We assume that exclusion of amplified *MYC*N copies in cultivated neuroblastoma cells, shown in this study, contributes to this process and represents a further hint of cell revertance. However, as mentioned above, exclusion of the amplified gene copies does not necessarily seem to be associated with F-cell formation as F-cell variants have been described in cell lines with hsr [18, 33, 34], as well as in non-*MYC*N amplified cell lines [9, 37–45]. Furthermore, it would be crucial to know whether the phenomenon of loss of *MYC*N amplification in the F-cells is reversible or not. Data on the so-called transdifferentiation between the different cell types in neuroblastoma cell lines are controversial. Although interconversion has been described to occur [10, 11, 20], some authors indicate the F-phenotype to be stable [46, 47] and that the F-cells show a finite life-span *in vitro* [19]. Summarising, we have shown evidence for spontaneous elimination of an amplified oncogene in cultivated neuroblastoma cells, resulting in the so-called F-cells that are known for their loss of the malignant phenotype. The question of whether the oncogene elimination can be enhanced by chemical agents, in addition to, for example, BrdU, are probably also of therapeutic interest.

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