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DNA PROTEIN FLOW CYTOMETRY OF DISSOCIATED CULTURES OF HUMAN ANAPLASTIC GLIOMAS. PATTERN OF PROLIFERATION AND DIFFERENTIATION, AND THE EFFECT OF A NEW CYTOSTATIC DRUG CLADRIBINE (2-CdA)

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A technique of protein-DNA flow cytometry was applied to characterize cell cycling, and to assess the cytotoxicity of cladribine (2-chloro-2'deoxyadenosine) toward seven dissociated cultures of human primary brain tumors (anaplastic astrocytoma and glioblastoma multiforme) grown *in vitro* from surgical biopsies. Control cytograms were suggestive of that a clonogeneic fraction of the cell population consists mainly of cells with low protein content, which do not require increase in protein content before entering the S phase of the cell cycle. Following 24 or 48 hours exposure to cladribine, 1 nM – 1 μM, no cytotoxic effect was evident in 4 cultures, whereas in two cases dose-dependent progressive block of the phase of the cell cycle was noted. In one case a massive cytotoxic effect resulted in disintegration of culture exposed to 100 nM of the drug. However, the treatment with cladribine was ineffective in a patient bearing the tumor which was the source for the last culture, suggesting that cytotoxicity *in vitro* may not be predictive of clinical response.

Key words: *glial tumors, cladribine (2-CdA), DNA-protein cytometry*

A technique of multiparameter flow cytometry allows to describe, in a population of cells, a statistical distribution of relative quantities of several cell constituents which are differentially stained and simultaneously measured. Furthermore, correlated measurements of these constituents on a cell-by-cell basis can be obtained (Steinkamp 1984; Darzynkiewicz, Traganos 1990). Some properties of particular cells may also be inferred from the population statistical data.

In flow cytometry, DNA content is one of the most frequently measured cell parameters. DNA measurements in a cycling cell population allow to identify cells in the G_o/G_1, and G_2/M phase of the cell proliferating cycle. Simultaneous measurement of DNA and RNA may contribute information concerning the relationship between cell cycling and differentiation. Protein content is usually correlated with that of RNA (Steinkamp 1984; Darzynkiewicz, Traganos 1990). DNA flow cytometry may also be employed to assess the effects and mode of action of cytostatic or cytotoxic drugs *in vitro*. For example, it has been shown with this technique that cytotoxicity of cladribine (2-chloro-2'-deoxyadenosine, 2-CdA) is phase-specific. In a leukemia line the drug induced apoptosis when cells were transversing the S phase of the cycle (Lassota et al. 1994).

Cladribine is a new antileukemic and immunosuppressive nucleoside proven considerably active in some leukemias and lymphomas (Beutler 1992; Grieb 1994). In a phase I study reported recently, treatment of seven cases of high grade malignant astrocytomas with cladribine resulted in two partial remissions lasting several months (Saven et al. 1993). In an on-going clinical study (Mazurowski et al. 1995) activity of the drug in some cases of anaplastic gliomas and astrocytomas was also suggested. These observations inclined us to perform *in vitro* assays of 2-CdA cytotoxicity against human malignant gliomas in dissociated culture. We hoped that responses to the drug *in vitro* may be predictive of its activity *in vivo*.

Material and methods

Tissue samples of anaplastic primary brain tumors obtained during open surgery or surgical biopsy performed in the Department of Neurosur-

gery, School of Medicine in Warszawa, were placed in the ice-cold MEM medium and transported to the tissue culture laboratory. Tissue specimens were cut into small pieces with a surgical knife, sieved through a 60 mesh screen of a cell dissociation sieve (Sigma) and seeded into plastic Falcon-type bottles containing MEM medium supplemented with l-glutamine and non-essential amino acids (Sigma), 10% fetal calf serum (Hungarpol) and antibiotics (penicillin, gentamycin, amphotericin C and nystatin). The bottles were kept in standard conditions for tissue culturing (37°C, 5% CO_2). After 24 hours cell debris was removed and the medium was changed. Later the medium was changed twice a week. During growth the cultures were examined daily with a reversed microscope.

The cells which became attached to the bottom of a flask during the first day proliferated as monolayers. When cells became sufficiently numerous, the parent culture was harvested with the use a cell scraper (Sigma), suspended in PBS and seeded into 6 different vessels. Daughter cultures were grown simultaneously for a few days, and later incubated with increasing final concentrations of cladribine (typically 1, 10, and 100 nM, in some cases also higher concentrations, up to 1 μM) for 24 and/or 48 hours. The drug was kindly supplied by Dr. Z. Kazimierczuk (Department of Biophysics, University of Warszawa). During incubation with cladribine the cultures were relatively sparse, allowing the assumption that they are in the logarithmic growth phase.

For flow cytometry the cultures were harvested with a cell scraper, centrifuged and suspended in PBS, and fixed in 75% ice-cold ethanol. Cell suspension was stained with two fluorescence dyes: 2,5-diphenylindode (DAPI, Molecular Probes) which binds specifically to the double-stranded DNA, and sulphorhodamine 101 (Molecular Probes) which binds unspecifically with proteins.

Suspension of stained cells was subjected to flow cytometric measurement with the use of a Partec PAS II flow cytometer equipped with HBO 100 mercury lamp as the excitation light source, an excitation filter UG-1 (for UV), a BG-38 thermal filter, and a TK-420 dichroic mirror. The fluorescence light was separated with a TK 520 dichroic mirror, and filters transmitting green ($\sim$460 nm) and red ($>$600 nm) light.

The cytograms were usually obtained for a population of ca. 20,000 cells. They were processed and recorded with a computer and programs of the Partec cytometer. The data were displayed in the form of cytograms and DNA and protein histograms (Fig. 1).

Results and discussion

A total of 24 cultures were initiated. The majority quickly stopped growing and degenerated before a sufficient number of cells was achieved. In some cultures microbial contamination developed. For these reasons the flow cytometric measurements could have been performed only in 7 cultures (five of which were histologically described as anaplastic astrocytomas, and two as glioblastoma multiforme)*.

A representative example of DNA-protein cytogram of anaplastic glioma in primary culture is shown in Fig. 2. In all measured cultures the control cytograms were qualitatively similar, especially in respect to the relative number of cells showing DNA content typical of the S phase (compare the cytograms on Fig. 1 left lower panel and on Fig. 2), and the relative number of cells disintegrating spontaneously, supposedly by apoptosis (i.e., cells showing lower than near-diploid DNA content).

The effects of the exposure to cladribine were diverse. In four cases no clear response was evident in concentrations up to 100 nM. In two cases changes were dose-dependent, as shown on sequence of histograms in Fig. 3. In one case (glioblastoma multiforme) virtually no cells were detected in culture following 48 h exposure to cladribine 100 nM, suggestive of a massive cytotoxic effect.

From heterogenous and frequently partially necrotized tissue specimens obtained during surgery only a few cells were usually able to attach to the surface and start to proliferate, and several cell divisions were necessary to obtain sufficient number of cells to perform cytometric measurements. For this reason our cultures were rather long-term than primary, and the frequency of failures is not surprising. It is known that only some 20% of primary cultures can be grown to permanent lines (Westermark et al. 1973).

In glioma cultures, a clear and consistent correlation has been reported between cell morphology and their long-term growth capacity. When a primary culture consists mainly of astrocyte-like cells, cellular cytoplasmic projections progressively increase in size and number, and cells seem to differentiate rather than multiply. Permanent lines can be established only from cultures in which spindle-shaped (less differentiated) cells dominate (Westermark et al. 1973; Nister, Westermark 1984). It is not clear why in so many cases

* The authors are indebted to Prof. H. Kroh for kind permission of using histopathological diagnostic protocols of tumors.

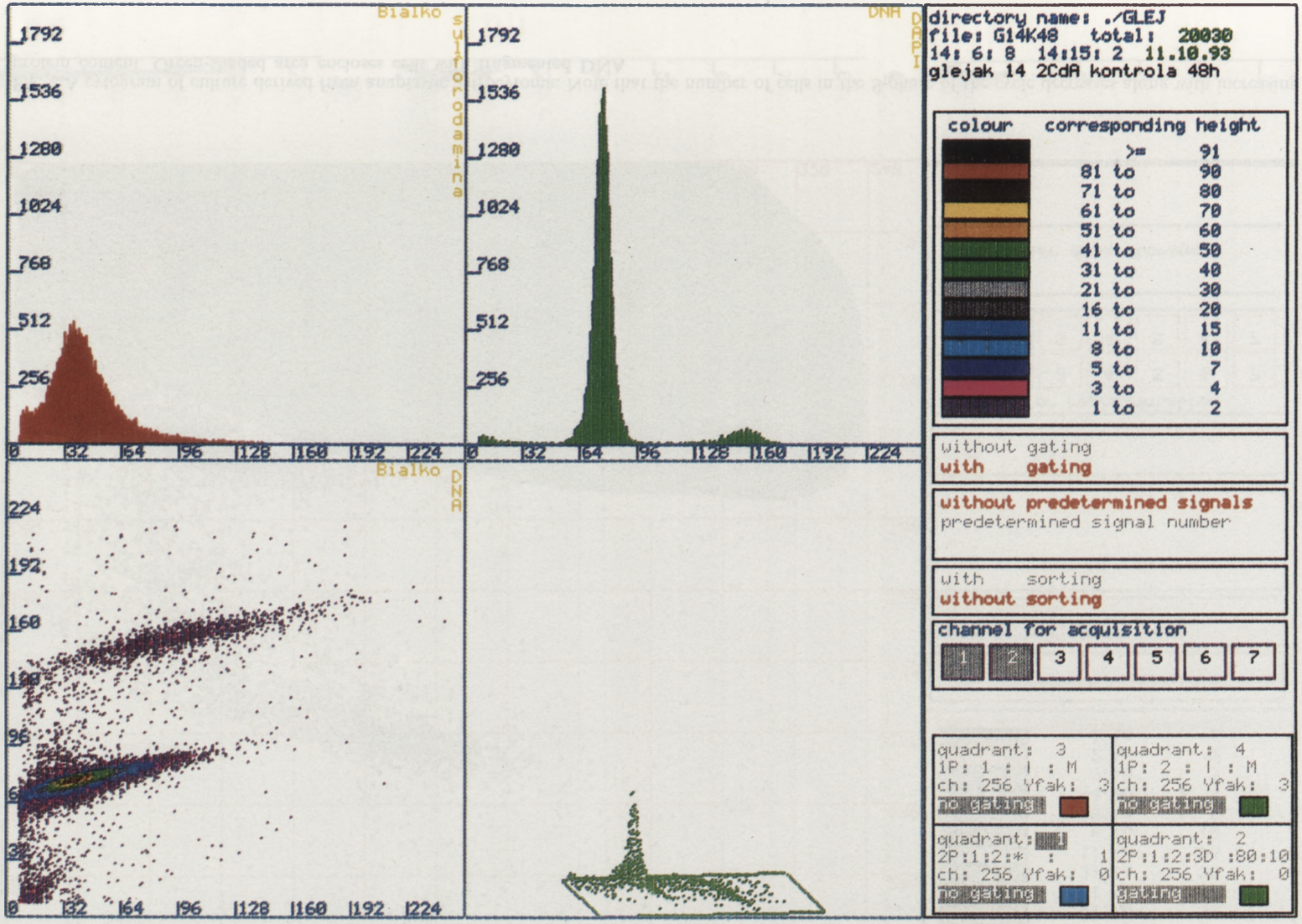

Fig. 1. Dissociated culture of anaplastic (grade 3) astrocytoma 48 hrs after repasaging. Printout of the data from a flow cytometric measurement. Total number of cells counted 20030. Left upper panel (red display), histogram of protein content (abscissa) vs. number of cells (ordinate). Protein content of cells increases along with channel number. Middle upper panel (green display), histogram of DNA content (abscissa) vs. number of cells (ordinate). DNA content increases along with channel number. Right upper panel, colour coding for cell-by cell cytogram display. Left panel, cell-by cell cytogram display. DNA content on abscissa, protein content on ordinate. Cells possessing the same DNA content locate in a cluster roughly parallel to the abscissa. Superimposition of cells having the same DNA and protein content is reflected by colour change. Middle lower panel, three dimensional display (not used). Right panel, display settings. Note that the cells are slightly hyperploid (2n DNA content reads at around channel 64 on the upper middle panel)

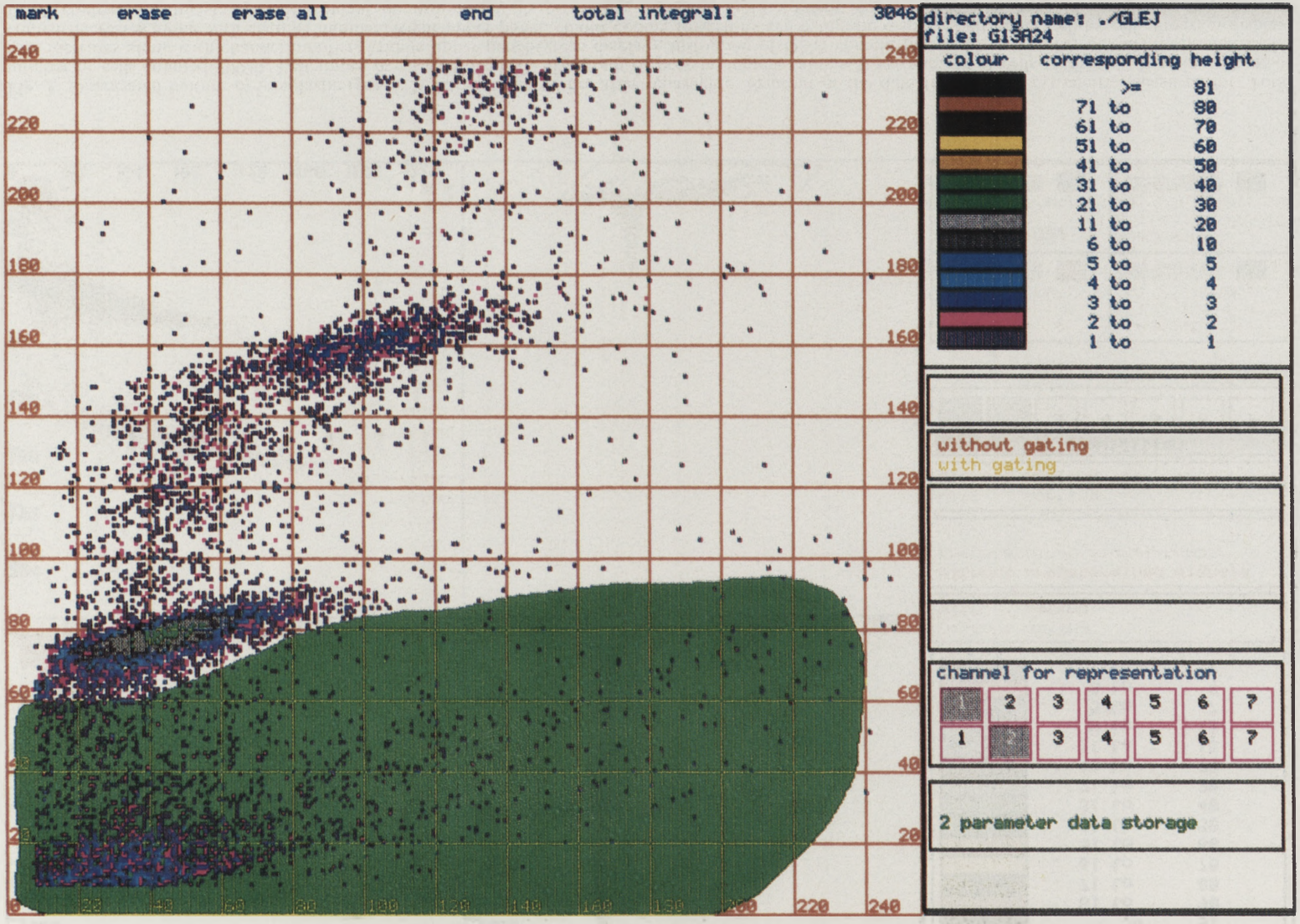

Fig. 2. A cytogram of culture derived from anaplastic astrocytoma. Note that the number of cells in the S-phase of the cycle decreases along with increasing protein content. Green-shaded area encloses cells with fragmented DNA

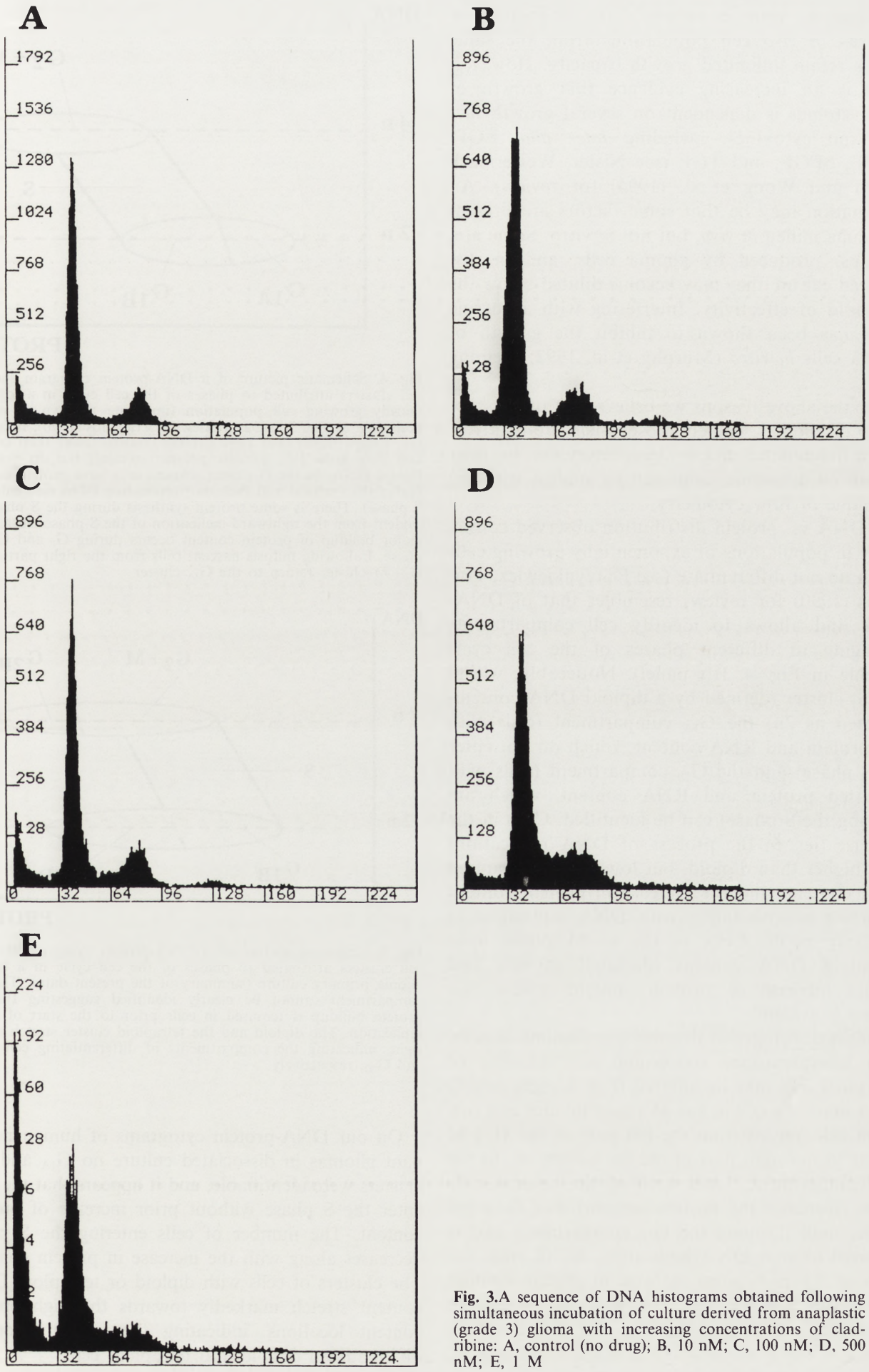

Fig. 3.A sequence of DNA histograms obtained following simultaneous incubation of culture derived from anaplastic (grade 3) glioma with increasing concentrations of cladribine: A, control (no drug); B, 10 nM; C, 100 nM; D, 500 nM; E, 1 M

glioblastoma cells in culture cease to proliferate, whereas *in vivo* cell populations from the same source retain unlimited growth capacity. However, there is an increasing evidence that growth of glioblastomas is dependent on several growth factors and cytokines, including *inter alia*, EGF, PDGF, bFGF, and TGF (see Nister, Westermark (1994) and Wong et al. (1994) for review). An explanation may be that some factors are present in glioma milieu *in vivo*, but not *in* vitro. Some are, perhaps, produced by glioma cells, and in dissociated culture they may become diluted below the threshold of effectivity. Interfering with autocrine loops has been shown to inhibit the growth of glioma cells *in vitro* (Murphy et al. 1992; Fleming 1992).

For the above reasons we believe that only a subgroup of gliomas, namely those which are relatively less differentiated and/or less dependent in their growth on autocrine loops can be studied with the technique of flow cytometry.

A DNA vs. protein distribution observed consistently in populations of exponentially growing cells which do not differentiate (see Darzynkiewicz, Traganos (1990) for review) resembles that of DNA-RNA and allows to identify cell compartments belonging to different phases of the cell cycle (scheme in Fig. 4, left panel). Noticeably, within the G_1 cluster (defined by a diploid DNA content, depicted as 2n) the G_{1A} compartment (cells with low protein and RNA content, which do not enter the S phase) and the G_{1B} compartment (cells with increased protein and RNA content, which are entering the S phase) can be identified. Cells in the S phase (ie., in the process of DNA replication) have higher than diploid, but lower than tetraploid DNA content, and the increase in protein content occurring concomitantly with DNA replication is relatively small. Cells in the G_2/M phase have tetraploid DNA content (depicted as 4n), and further increase of protein content within this cluster is evident.

Although cytograms describe populations of cells, some interpretations concerning the behavior of individual cells may be inferred (Fig. 4, right panel). Upon mitosis a cell in the M phase divides and two ascent cells return from the left part of the G_2+M cluster to the right part of the G_1 cluster, ie., to the G_{1A} compartment. Later each of the nascent cells grows (increases the protein content), but does not divide, until it enters the G_{1B} compartment and is prepared to start DNA replication, ie., to enter the S phase. There is some increase in protein content during the S phase, but the majority of protein accumulation occurs in the G_{1A}, and in the late G_2 phase.

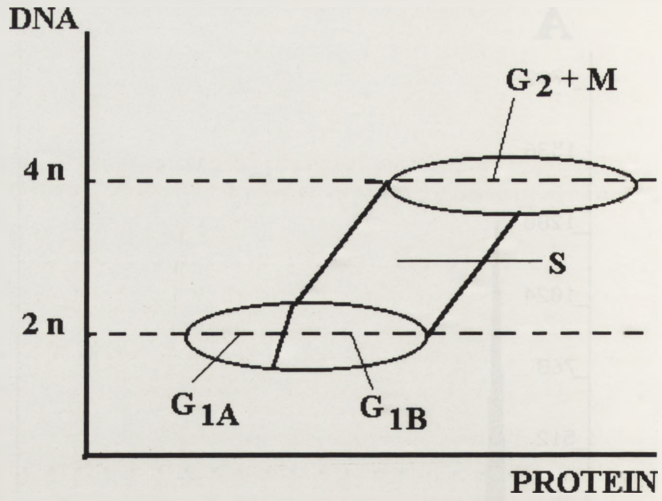

Fig. 4. Schematic picture of a DNA-protein cytogram showing cell clusters attributed to phases of the cell cycle in a logarithmically growing cell population (imitating the typical picture according to Fig. 3 in Darzynkiewicz, Traganos (1990). Inside the G_{1A} compartment cells have protein content lower than critical, and they grow (ie., increase protein content) but do not synthesize DNA. In the G_{1B} compartment cells have protein content higher than critical and they start replicating DNA (ie., enter the S phase). There is some protein synthesis during the S phase, as evident from the rightward inclination of the S-phase cluster, but major buildup of protein content occurs during G_1 and G_2+M phase. Following mitosis nascent cells from the right part of the G_2+M cluster return to the G_{1A} cluster

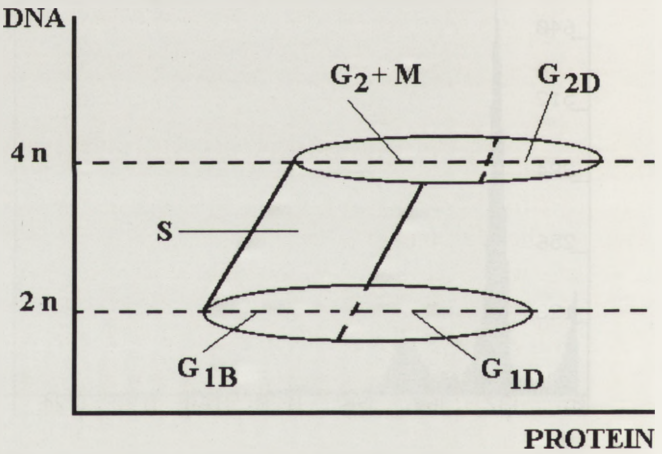

Fig. 5. Schematic picture of a DNA-protein cytogram showing cell clusters attributed to phases of the cell cycle in a human glioma primary culture (summary of the present data). The G_{1A} compartment cannot be clearly identified suggesting that no protein buildup is required in cells prior to the start of DNA replication. The diploid and the tetraploid cluster stretch to the right, indicating the compartments of differentiating cells, G_{1D} and G_{2D}, respectively

On our DNA-protein cytograms of human malignant gliomas in dissociated culture no G_{1A} and G_{1B} clusters were identifiable, and it appears that the cells enter the S phase without prior increase of protein content. The number of cells entering the S phase decreases along with the increase in protein content. The clusters of cells with diploid or tetraploid DNA content stretch markedly towards the high protein content locations, indicating the accumulation of differentiated diploid and tetraploid cells.

In most cell populations the increase in protein (and in RNA) content is a prerequisite for entering the S phase, in agreement with classical concept of the "critical mass", attainment of which is required to trigger DNA replication (Killander, Zetterberg 1965a,b). It is possible that cell division (mitosis) is followed by turn-out of expression of S-phase specific proteins subserving DNA replication, such as eg., ribonucleotide reductases (Eriksson et al. 1984) and *de novo* synthesis of these proteins may underlay the "critical mass" requirement. However, in our primary glioma cultures the cells with the lowest protein content preferentially enter the S phase. These small cells double their protein content along with replicating DNA and they keep proliferating most vigorously (see scheme on Fig. 5, right panel). It is possible that the expression of S-phase specific proteins is not turned off at cell division in these clonogeneic cells. When they start to accumulate protein (hence to differentiate), they gradually loose their proliferative potential. Although this observation could not be quantitated with the equipment currently in our hands, it is evident from the cytograms and may be interpreted as the evidence that in malignant glioma cells the commitment to differentiation does not exclude the commitment to proliferation on an "all-or none" basis.

Cladribine is known to inhibit DNA synthesis both in cells (Griffig et al. 1989) and in subcellular model systems (Hentosh, Grippo 1994). The mechanism at the cellular level may involve inhibition of ribonucleotide reductases by cladribine triphosphate and/or direct interference with the process of DNA replication. In any case prior activation of the drug by enzymatic phosphorylation is a prerequisite (see Grieb 1994, for review). The enzymes phosphorylating cladribine are cytosolic deoxycytidine kinase (dCK) and mitochondrial deoxyguanosine kinase (dGK) (Eriksson et al. 1994). Preliminary attempts to assay cladribine-phosphorylating activity in specimens of anaplastic gliomas did not produce impressive results. In the study of Saven et al. (1993) no such activity was found and the concentration of dCK polypeptide was minute. We (Grieb et al. 1993) were able to assay cladribine-phosphorylating activity of dCK and also dGK in glioma samples, and some of them contained activity markedly higher that in neighbouring non-malignant brain tissues; yet, phosphorylation of the drug proceeded consistently at least one order of magnitude slower than in lymphocytes, model target cells of cladribine cytotoxicity.

In two of our cultures, incubation with increased concentrations of cladribine resulted in a dose-dependent block of the S phase, a cytostatic rather than cytotoxic effect. The block appeared at the late S phase and it was progressing toward the early S phase as the drug concentration increased. At the concentration of 1 μM picture of cytotoxicity (induction of apoptosis) was observed, with a deeply reduced number of cells with DNA content characteristic of the S phase and, in particular, of the G_2+M phase, and the appearance of a large number of cells with less than diploid DNA content (hence, cells with fragmented and partially degraded DNA). The result is suggestive of that cells transversing the S phase are the most prone to enter apoptosis induced by cladribine, similarly as it is in the case of leukemia cells (Lassota et al. 1994).

One might expect that it is the cytotoxic effect which has relevance to the clinical activity of antineoplastic drug. Unfortunately in the clinical setting nanomolar rather than micromolar concentrations of cladribine are available to tumor tissues (Liliemark, Juliusson 1994). Clinical treatments with cladribine last five to seven days, compared to one or two days of exposure employed in the present study. However, the cells may concentrate the drug in its phosphorylated forms, and cytotoxic concentrations may be achieved intracellulary at longer exposure times. During treatment with cladribine the concentration of cladribine phosphates retained inside chronic lymphocytic leukemia cells is approximately two orders of magnitude higher than that in patient's plasma (Liliemark, Juliusson 1994). Furthermore, subcytotoxic concentrations of cladribine may inhibit cellular cytokine production, as it was shown for IL-6 cultured monocytes (Carrera et al. 1982). Such a mechanism may contribute to cytostatic effect *in vivo*, but is not detectable with our experimental approach.

Overall, *in vitro* data provide evidence that cladribine is active against some malignant gliomas in dissociated culture. The drug induces the dose-dependent S phase block, and cell death at higher concentrations. However, the properties of cultured glioma cells substantially differ from those of cells in parent tumor *in vivo* and the results from *in vitro* experimentation shall be interpreted with great caution. Interestingly, a patient whose cultured glioma cells responded *in vitro* by massive death following 48 h exposure to 100 nM cladribine, completely failed to respond to the treatment (no progression-free survival was noted). We do not believe, that at least in the case of cladribine, DNA flow cytometry will provide a useful information concerning susceptibility of gliomas to treatment. At the moment, carefully designed clinical trials still remain the ultimate proof of drug anti-tumor effect.

Badania zdysocjowanych hodowli ludzkich złośliwych guzów glejopochodnych metodą cytometrii przepływowej DNA-białko. Obraz proliferacji i różnicowania komórek oraz wpływ nowego leku cytostatycznego cladribine (2-CdA)

Streszczenie

Technika cytometrii przepływowej DNA-białko została zastosowana do zdysocjowanej hodowli pierwotnych guzów mózgu (anaplastycznych gwiaździaków i glejaków wielopostaciowych) celem scharakteryzowania cyklu komórkowego i cytotoksyczności cladribine (2-chloro-2'-dezoksyadenozyny). Badaniom poddano siedem hodowli wyprowadzonych z biopsji neurochirurgicznych. Cytogramy kontrolne sugerowały, że komórkami klonogennymi są głównie komórki o niskiej zawartości białka, które nie muszą zwiększać zawartości białka przed wejściem w fazę S cyklu komórkowego. Po 24-48 godzinach ekspozycji na cladribine w stężeniach od 1 nM do 1 μM w 4 przypadkach stwiedzono brak efektu, podczas gdy w dwóch innych wystąpił zależny od stężenia postępujący blok fazy S cyklu komórkowego. W jednym przypadku wystąpił silny efekt cytotoksyczny, który doprowadził do śmierci hodowili po ekspozycji na 100 nM leku. Jednak u pacjenta, którego guz był źródłem materiału do tej ostatniej hodowli leczenie przy pomocy cladribine okazało się całkowicie nieskuteczne. Sugeruje to, że cytotoksyczność *in vitro* nie musi zapowiadać odpowiedzi klinicznej.

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