Growth Kinetics of the G2-Phase of Ehrlich Ascites Tumor Cells, Separated from Anaerobically Treated Asynchronous Cultures

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Cell cycle progression of G2 fractions (75−80% G2 (4C) cells) from 8 h anaerobically cultured asynchronous hyperdiploid Ehrlich ascites tumor cells strain Karzel, separated by centrifugal elutriation, was studied after reaeration by flow cytometric methods, including the BrdU-H33258 technique and dual parameter measurements. Analyses of the growth kinetics demonstrated that one fraction of the cell population proceeds through a normal cell cycle (2C → 4C) with a generation time of about 20 h. Another portion entered a new cycle (4C → 8C) to form cells with a DNA content up to 8C; mono-, bi- and polynucleate cells could be detected. After 15 h aerobic recultivation of the anaerobically cultured G2 cells, a fraction containing 80−85% with a DNA content of > 4C was separated. On recultivation, these cells pass a 4C → 8C division cycle with a generation time of about 10 h, and a G1 period of less than 4 h.

Biochemical and nutritional factors that control the growth and division of tumor cells, including macromolecular synthesis, are very complex. Within solid tumors there exist gradients of cellular nutrients, for instance, of oxygen, glucose and amino acids [1]. Hypoxic tumor cells have long been recognized as populations that are potentially resistant to radiotherapy [2] and also to chemotherapy [3]. In experimental tumors hypoxic cells can comprise more than 80% of the viable cells [4]. Some tumors may reoxygenate under certain conditions but the extent and time-course of reoxygenation depend on the respective tumor [5].

During the last years we have been interested in how extreme hypoxia effects tumor cell proliferation and how cycles of hypoxia and reaeration affect their viability and proliferation kinetics. Our investigations on the proliferation kinetics of *in vitro* grown asynchronous hyperdiploid Ehrlich ascites tumor cells under exclusion of oxygen have shown that, cells which are in the G1 stage at the beginning of deprivation of oxygen continue to synthesize protein and increase in volume; they accumulate in the late G1 period but do not enter the S-phase under these conditions. During the first 6−8 h of exclusion of oxygen, late S cells enter the G2 period but do not divide; most of the S-cells remain in their compartment during anaerobiosis. Cells present in G2 at the

beginning of the experiment undergo mitosis and their division accounts for the increase in cell number under anaerobic conditions [6, 7].

In the present work we have studied the growth characteristics after reaeration of those cells, which were in the G2 compartment after a period of 6−8 h of deprivation of oxygen; these cells completed their DNA-synthesis under exclusion of oxygen. Their cell cycle progression after reaeration is of special interest since DNA-synthesizing cells are generally more sensitive to deprivation of oxygen [8, 9]. On the other hand, G2-phase cells which have completed replication seem to be less vulnerable than S cells to the critical transition from anaerobic to aerobic conditions. Our experiments have shown, that the growth kinetics of these cells are characterized by an over-replication of DNA and the formation of polyploid cells, which seem to enter an atypical division cycle of short doubling time.

Materials and Methods

All chemicals, buffers and media components were of the purest grade available from Merck (Darmstadt), Serva (Heidelberg), Sigma (Munich) and Boehringer (Mannheim). Horse serum was a gift from the Behring Werke (Marburg); it was essentially free of mycoplasma. Microcillin was a gift from Bayer (Wuppertal-Elberfeld). [Methyl-^{3}H]thymidine (spec. act. 42 Ci/mol) was from Amersham (Braunschweig); ethidium bromide, 4,6-diamidino-2-phenylindol (DAPI) and bromodeoxyuridine (BrdU)

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were from Serva, bis-benzimid H33258 was from Riedel de Haen (Hannover); Sulforhodamin 101 (SR 101) was from Sigma; and Argon/CO_2 = 95:5 was from Messer (Griesheim).

Cell cultivation and separation

Hyperdiploid EAT cells, strain Karzel serially passaged in female NMRI mice, were grown in suspension culture using Eagle's medium containing 10% horse serum, 10 mM Hepes buffer, microcillin (532.0 mg/l) and streptomycin (30 mg/l). The nutrients of the medium were sufficient for a 24 h culture period. Growth was determined with the particle counter of the flow cytometer Partec PAS II or by counting the cells in a Neubauer chamber. Viability of the cells was assessed by dye exclusion with 0.1% nigrosin. After 10−12 h cultivation at 37 °C (first passage *in vitro*) and harvesting, cells were transferred to fresh culture medium. To obtain anaerobic culture conditions the flasks were continuously flushed with Argon/CO_2 = 95:5 at a rate of 60 ml/min. The gas passed through a heated oxygen-adsorbing catalyst (R 3-11/M 3610, BASF, Ludwigshafen) and was then humidified by bubbling through a water column at 37 °C.

Fractions with a high content of G2 (4C)- and > G2 (> 4C) cells were obtained by centrifugal elutriation [10]. The method permits the collection of cells at different stages in the cycle by exploiting the increase in cell volume during the cycle and accompanying changes in the sedimentation rate, *i.e.* a JE-6 elutriator rotor with standard chamber (Beckman, Munich). In a typical experiment the rotor was loaded with approximately $1.2−1.5 \times 10^8$ cells (4 × 50 ml cultures) at a rotor speed of 1540 rpm and a flow rate of the elutriation medium (Hank's solution containing 5% horse serum) of 15 ml/min. By increasing the flow rate (FR), fractions containing 80−85% G2 cells (DNA = 4C, FR = 25−27 min) and up to 80% octaploid cells (DNA = 8C, FR = 40−45 ml/min) could be separated. The enrichment of cells at a certain stage of the cycle depends on the phase distribution of the cell populations at the beginning of the centrifugation.

Cell cycle analysis

Sequences of DNA histograms were used for monitoring the cell cycle distribution of the cultures. After washing the cells with Hank's solution, they were fixed by suspension in 96% ethanol and stored at −18 °C. After treatment with 0.1% RNAse (Serva, DNAse free) at 37 °C for 2 h, cells were stained with ethidium bromide (10 mg/l in 50 mM Tris buffer, pH 7.5). Fluorescence of stained DNA was measured with an ICP 11 flow cytometer (Phywe, Göttingen) equipped with a high pressure mercury lamp excitation filters BG 38 + BG 3 and a barrier filter GG 550. Bisbenzimid H33258 stained DNA was measured with the Partec PAS II System (see below).

Ethidium bromide staining of cells was combined with the BrdU-H33258 technique of flow cytometry [11, 12], to analyze the progression of the cells through the cell cycle. The latter technique uses the quenching of H33258 fluorescence by BrdU substitution for thymidine, thus leaving every cell in its fluorescence compartment during progression through the S compartment until division. For further details see *loc. cit.* [11] and [12].

For application of the BrdU-H33258 technique, cells were grown in the presence of 1.3×10^{-4} M BrdU and 1.2×10^{-4} M deoxycytidine (to overcome the cytotoxic effects of bromodeoxyuridine). At appropriate intervals, about 10^6 cells were fixed as described above and treated with 100 µl 0.1% RNAse at 37 °C. After 2 h the RNAse treatment was stopped with 0.5 ml of 0.5% Tween 20, in 0.2 M sodium citrate, pH 7.0; 2 ml H33258 (10 mg/l 25 mM Tris buffer, pH 7.0, 25% in ethanol) is added, with sample measurement after 30 min.

The simultaneous analysis of DNA and protein was performed as described by Stöhr [13] with 4,6-diamidino-2-phenylindol (DAPI) for DNA and sulforhodamin 101 (SR 101) for protein staining. About 10^6 cells were fixed with ethanol and stored at −18 °C. Cell staining involves the addition of 1 ml SR 101 solution (3 mg SR 101 in 100 ml 0.18 M Tris buffer, 0.18 M NaCl, pH 7.0) and 30 µl DAPI solution (10 mg DAPI in 100 ml of the same buffer). The samples may be stored for several days in the refrigerator before measurement. Stained cells in the reagent solutions were analyzed in the Partec PAS II System (Switzerland) equipped with a high pressure mercury HBO 100 W/2 lamp; excitation filter KG 1, BG 38, UG 1; TK 420, barrier filters G 435 and RG 590.

For simultaneous analysis of DNA and RNA content the following procedure was applied (see also Darzynkiewicz *et al.* [14]); after washing 2 times with

cold Hank's solution about 10^5 cells were fixed in 10 ml cold ethanol and stored at $-18\,°C$ for 2 h. Thereafter, the cells were washed with 0.9% NaCl solution, pelleted, suspended in 0.2 ml culture medium and mixed with a cold solution containing 0.1% (vol/vol) Triton X 100, 0.08 N HCl and 0.15 M NaCl. After 25 sec gently shaking in the cold, cells were stained by the addition of 1.2 ml cold acridine orange (AO) solution (20 μM AO (chromatographically pure), 1 mM EDTA, 0.15 M NaCl in 0.2 M phosphate — 0.1 M citrate buffer pH 6.0). After 5–20 min of equilibration the fluorescence intensities of individual cells were measured in the flow cytometer Partec II. The intensity of the green fluorescence of the AO-DNA complex was measured between 515 and 560 nm, the red fluorescence of AO-RNA complex was measured between 590–630 nm. Excitation filters KG 1, BG 38, BG 12; TK 500; barrier filters GG 515 and RG 630 were used.

All data were stored on hard disc and analyzed by software as described in *loc. cit.* [15]. Dual parameter measurements were essentially evaluated as described by Ellwart *et al.* [16].

Results

Growth kinetics of tetraploid (4C) cells

Ehrlich ascites tumor cells harvested from the peritoneal cavity of female NMRI mice 5–6 days after inoculation, were cultured anaerobically for 8 h, after a first *in vitro* passage of 13–15 h under standard conditions. By optimizing the separation procedure, it was possible to obtain from these cultures by centrifugal elutriation, fractions with 80–85% G2 cells (DNA = 4C) which in most cases also contained cells with a DNA content > 4C. The relation between phase composition and flow rate of elutriation-medium is illustrated in Fig. 1.

In order to first obtain information on the effects of deprivation of oxygen on the proliferation kinetics of the G2-phase cells after reaeration, these fractions were recultivated under standard conditions and their cell cycle progression analyzed by flow cytometric methods. The results of a typical experiment revealed that, one part of the cells passes a $2C \rightarrow 4C$ cycle with a reduced generation time as compared to asynchronous standard cultures (see below); a second part starts to synthesize DNA without a preceeding division and moves into the 8C compartment. Obviously, these cells traverse a $4C \rightarrow 8C$ division

cycle. Apparently the cell cycle distribution is the result of two parallel proceeding cell division cycles. The 4C peak results from the fluorescence signals of G2 cells of the first division cycle ($2C \rightarrow 4C$) and of G1 cells of the second cycle ($4C \rightarrow 8C$). The histograms also demonstrate that some of the G2 cells do not leave their compartment or enter mitosis only very slowly after reaeration.

Flow cytometric analyses of more than 15 recultivated G2 populations have further shown, that the fraction of cells leaving the normal $2C \rightarrow 4C$ cycle and over-replicating DNA decreases with an increasing proportion of G1- and S-phase cells in the G2 population at the beginning of recultivation. Thus, after 19 h recultivation of a G2 population consisting of 8% G1-, 38% S- and 54% G2 cells 20% cells with DNA content > 4C could be detected, whilst starting with a culture of 3% G1-, 19% S- and 78% G2 cells the fraction of over-replicating cells after the same time was 43%.

The doubling time of the anaerobically treated G2 cells after reaeration was estimated by applying the BrdU-H33258 method of cytometry. A comparison of a series of total and quenched DNA histograms of a recultivated G2-cell population demonstrated: that after 16 h nearly all G2 (4C) cells have left their compartment; 4 h later one portion of these cells has already passed the next cell cycle as can be concluded from the emergence of a G1/2 (1C) peak in the 20 h DNA histogram; the same is true for the late S-phase cells, which appear at the left of the G1 (2C) peak of the quenched histograms; and late S- and G2-phase cells apparently reveal the same proliferation characteristics. Their generation time can be estimated to be between 16 and 20 h. The fluorescence of cells
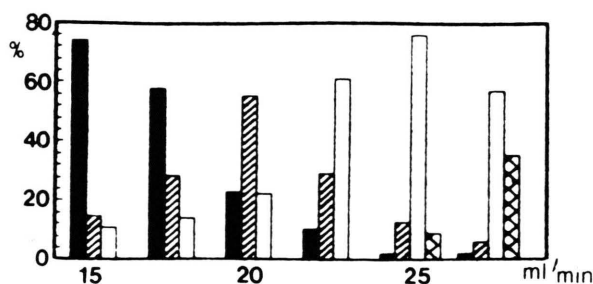

Fig. 1. Fractionation of 8 h anaerobically grown EAT cells by centrifugal elutriation. Relation between DNA content of the fractions and flow rate of the elutriation medium. ■ 2C-cells, ▨ > 2C- — < 4C-cells, □ 4C-cells, ◪ > 4C-cells; for further details see Material and Methods.

with a DNA content $> 4C$ is nearly completely quenched if they are grown in the presence of BrdU; over-replication of a significant proportion of the G2-phase cells can, however, be followed in the total DNA histograms.

Light micrographs of smears with $80-85\%$ cells with a DNA content of $> 4C$ have revealed $20-25\%$ cells with two nuclei. Flow cytometric analysis after hypotonic treatment displayed the portion of binucleate cells as 37% (see Fig. 2).
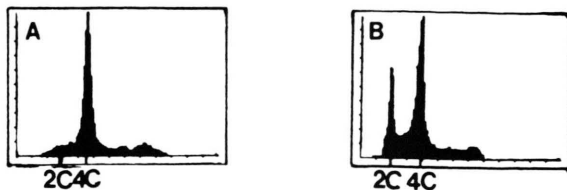

Fig. 2. DNA histograms of G2 (4C)-populations (A) before and (B) after hypotonic treatment of the probes. 4C-cells with two nuclei give nuclei with DNA = 2C after disintegration under hypotonic conditions. Unfixed cells were stained with Propidium jodide in hypotonic medium (50 µg/ml Propidium jodid in 0.5% sodium citrate solution, addition of 100 µl 0.1% RNAse).

Growth kinetics of octaploid (8C) cells

Cells with a DNA content of $> 4C$ were separated from $13-15$ h aerobically recultivated G2 (4C) fractions by centrifugal elutriation. The relation between phase composition and flow rate of the elutriation medium is illustrated in Fig. 3. It is possible to obtain fractions with up to 85% of cells possessing a DNA content $> 4C$ (S* + G2M* cells), though the yields are low. In the first elutriation step for separation of G2-enriched populations about 10% of the anaerobically grown asynchronous cultures were recultivated; by the second centrifugation maximally 15% of the $13-15$ h aerobically recultivated G2 cells with a DNA content > 4 were separated.
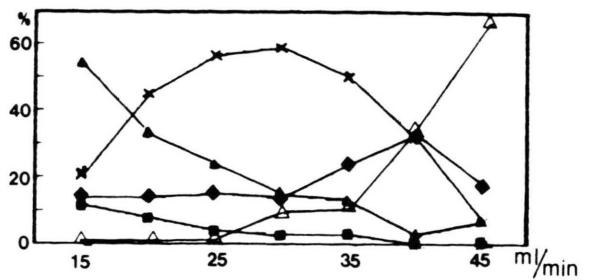

Fig. 3. Fractionation of $13-15$ h aerobically cultured G2-(4C) enriched populations, which were obtained from 8 h anaerobically treated asynchronous EAT cells. Relation between DNA content of the fractions and flow rate of elutriation medium. ■ 2C-cells, ▲ $> 2C- - < 4C$-cells, × 4C-cells, ◆ $> 4C- - < 8C$-cells, △ 8C-cells.

DNA histograms of a G2 fraction separated from anaerobically grown asynchronous cultures, of the same fraction after 15 h recultivation and of the 8C cells separated from the latter population are depicted in Fig. 4. As is shown in the light micrographs of smears of these cells (Fig. 5), the structure of the nuclei of the 8C-cell fraction is rather heterogeneous. Cells with only one large nucleus as well as with two and more nuclei can be discerned. The proportion of multinucleate cells was estimated to be 35% (30% bi-, 4% tri- and 1% tetranucleate) by microscopic counting. By cytometric analysis of the 8C fraction before and after hypotonic treatment about 45% multinucleate cells were detectable: Under hypotonic conditions, a significant portion of 8C cells disintegrate forming 4C nuclei (decrease of the 8C peak, increase of the 4C peak).

DNA histograms of 8C cells grown under standard conditions and in the presence of BrdU have proved that 8C cells divide and enter a $4C \rightarrow 8C$ division cycle. After 10 h a 2C peak appears in the quenched histograms indicating, that 8C cells have divided to form 4C cells, which after this time period, have already passed the next cycle with halving of the
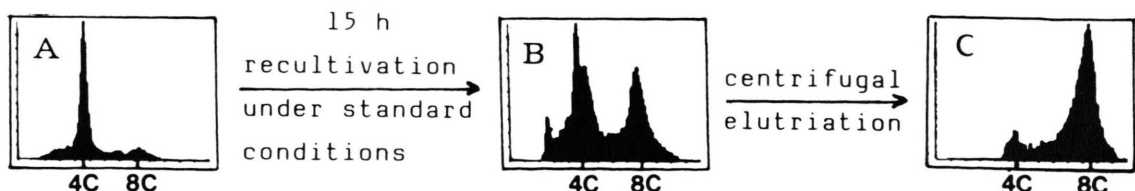

Fig. 4. DNA-histogram of a G2 (4C)-fraction, separated from 8 h anaerobically grown EAT cells (A), of the same G2-fraction after 15 h recultivation (B), of the 8C-fraction separated from this population (C).
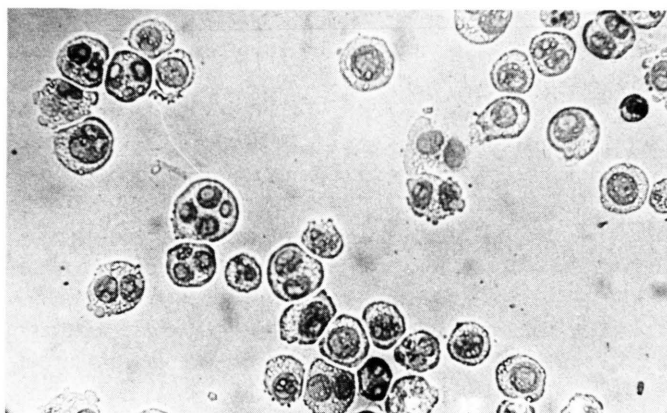
Fig. 5. Smear of Feulgen-stained population with high content of 8C cells. Cells with one large nucleus and multinucleate cells can be detected.

mean fluorescence. This cycle takes less than 10 h. Possibly some of the 4C cells enter the 2C → 4C cycle, which may be concluded from fluorescence signals at the left side of the 4C peak or to the left of the 2C peak of the quenched histograms. Since it is well known, that the length of the G1 period of the cell cycle is the most variable, we suggest that the 4C → 8C cycle has a very short G1 period. This assumption is supported by autoradiographic studies.

A typical pulse-labelling experiment started with a cell population of the following DNA distribution: <4C(= S): 14%, 4C(= G2 + G'1): 4%, >4C−<8C (= S'): 27%, 8C (= G'2): 55%. At the beginning of cultivation the labelling index was 40%, after 4 h 29%, after 8 h 33% and after 12 h 45%. An increase of the labelling index within the time period of 4−12 h is only possible if the 8C cells begin DNA synthesis shortly after they have divided. Labelling of the cells would decrease during this time if they had to pass a G1 period of more than 4 h. Within the first hours the labelling index decreases (40% → 29%) because S' cells (DNA > 4C and < 8C) and S cells (DNA < 4C) leave their compartment. G2 cells, which enter the next cycle have not passed the short G1 period of about 3 ± 1 h at this time. Cells with a DNA content of < 4C (S cells) (14%) and > 4C−< 8C (S' cells) (27%) make together 41% according to the flow cytometric analysis; this value is in good agreement with the labelling index of 40% at the beginning of the experiment.

We have further been interested as to whether division of 8C cells occurs mainly *via* mitosis or cytokinesis of multinucleate cells. To answer this question, cells were grown in the presence of 1 μM
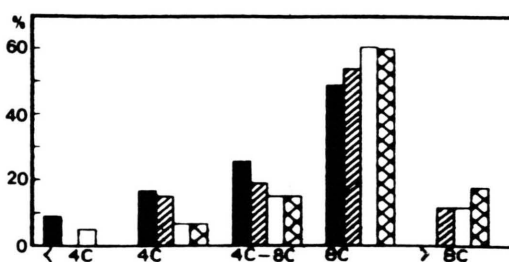

Fig. 6. Cell-age distribution of an 8C enriched population over a period of 24 h grown in the presence of 1 μM Colcemid. Result of flow cytometric analysis. ■ 0 h, ▨ 5 h, □ 11 h, ▧ 24 h. Accumulation of 8C cells as a result of inhibition of mitosis by the drug.

colcemid. The results of flow cytometric analysis of cells grown in the presence of this drug are summarized in Fig. 6. The cell cycle distribution reveals a significant accumulation in the 8C compartment. However, under standard conditions (without colcemid) the DNA histograms demonstrated a decrease of 8C cells within 24 h. These data allow the conclusion, that 8C cells can pass a colcemid inhibitable mitosis. This is confirmed by light microscopic micrographs, which demonstrate multipolar mitoses (not shown).

Dual-parameter flow cytometry
Simultaneous measurement of DNA/RNA
and DNA/protein

In order to obtain information on the metabolic state and capacity of anaerobically pretreated G2 cells after recultivation and of 8C cells separated from the recultivated G2 populations, we have ap-

plied dual parameter flow cytometry, which allows to correlate cellular RNA and protein content in individual cells with their position in the cell cycle. This technique is particularly applicable to studies in which protein (RNA) content and DNA to protein (DNA to RNA) ratios may provide information on the metabolic state of cell populations in various phases within the cell cycle.

A typical cell-age distribution of DNA to protein and DNA to RNA in G2-enriched cells after recultivation is illustrated in Fig. 7A and B. The intensity of H33258 fluorescence is proportional to the DNA content per cell and makes it possible to classify cells within the G1-, S- and G2M-phases of the cell cycle, while the fluorescence of SR101-stained protein is proportional to the protein content of the cells. The DNA histograms reveal a much better resolution of the cell-age distribution after staining with DAPI than with acridine orange, which was applied for staining DNA and RNA. Analyses of the fluorescence pattern indicate that a progressive increase in protein (RNA) occurs during the cell cycle; this is true for the 2C → 4C cycle as well as for the 4C → 8C cycle. G2 (4C) cells have nearly twice as much protein (RNA) as G1 (2C) cells. This ratio is also correct for 4C and 8C cells. The scattergrams are typical for two parallel proceeding cell cycles. They seem to confirm the existence of a threshold of the protein and RNA content in the G1 (2C) compartment, which is required for cells to be able to initiate DNA synthesis as is proposed by several authors [17]. Threshold values of the protein and RNA content can be detected in the 0, 3 and 6 h histograms of Fig. 7A and B.

DNA- and RNA-profiles and, especially, the scattergrams of dual parameter flow cytometric measurements of the cycle progression of 8C cells reveal no significant threshold values between 4 and 24 h. G'1 (4C) cells enter directly the S' phase without accumulation of protein or RNA to a minimal level. 4C cells with an extreme protein content do not seem to initiate DNA synthesis. The histograms further confirm the rapid progress of the cells through the cycle (histograms not shown).

Discussion

In previous investigations we have compared cell cycle progression of EAT cells under aerobic and anoxic conditions and of cells reaerated after a period of anoxia [18−22]. In the present communica-

tion we have pursued these investigations with a study on the proliferation kinetics of G2 (4C) cells, which were separated from 8 h anaerobically grown asynchronous cell cultures. The growth behaviour of a portion of these cells is obviously characterized by a propensity for over-replication, while another part passes a normal 2C → 4C cycle lasting 20 ± 2 h. It was possible to separate from these cultures − though in low yields − fractions consisting of 80−85% cells with a DNA content > 4C; on recultivation they traverse a 4C → 8C cycle in about 10−12 h. These conclusions were drawn from a comparison of T-DNA and BrdU-DNA histograms. From our data we further conclude, that the number of G1 and S-phase cells, present in the G2-enriched fraction at the beginning of recultivation, influences the portion of over-replicating cells. Possibly, this phenomenon is a special case of the well known interaction of cells of different compartments of the cell cycle [23]. In some cases it was possible to identify factors, which mediate these interactions [24].

As was postulated by several authors [17, 25−27], the initiation of DNA synthesis requires a threshold of protein- and RNA-content. This could also be demonstrated by dual parameter flow cytometry for the 2C → 4C cycle of the anaerobically treated G2-phase cells. In contrast, over-replicating cells begin directly DNA synthesis without perceptible thresholds of protein- or RNA-content. In the normal 2C → 4C cycle cells do not initiate DNA synthesis after they have spent a certain time in the G1 compartment but only after their protein (RNA) content has attained a critical level. This does not seem to be true for the 4C → 8C cycle; in these cells the rate (probability) of exit from the G1 compartment is not correlated with their protein-(RNA) content.

It is well established, that the karyotype of EAT cells is unstable. The first studies on endomitotic reduplication of these cells *in vivo* stem from Levan and Hauschke [28], who have already demonstrated that different mechanisms can account for the emergence of polyploid cells. Formation of binucleate cells occurs by cytoplasmatic fusion of two mononucleate cells or by karyokinesis without cytokinesis; tetranucleate mega cells emerge if this mode of division is repeated. In most cases, binucleate cells seem to be intermediate stages in the development of polyploidy [29, 30].

Concerning the molecular mechanisms involved, possibly a population of cells with increased DNA
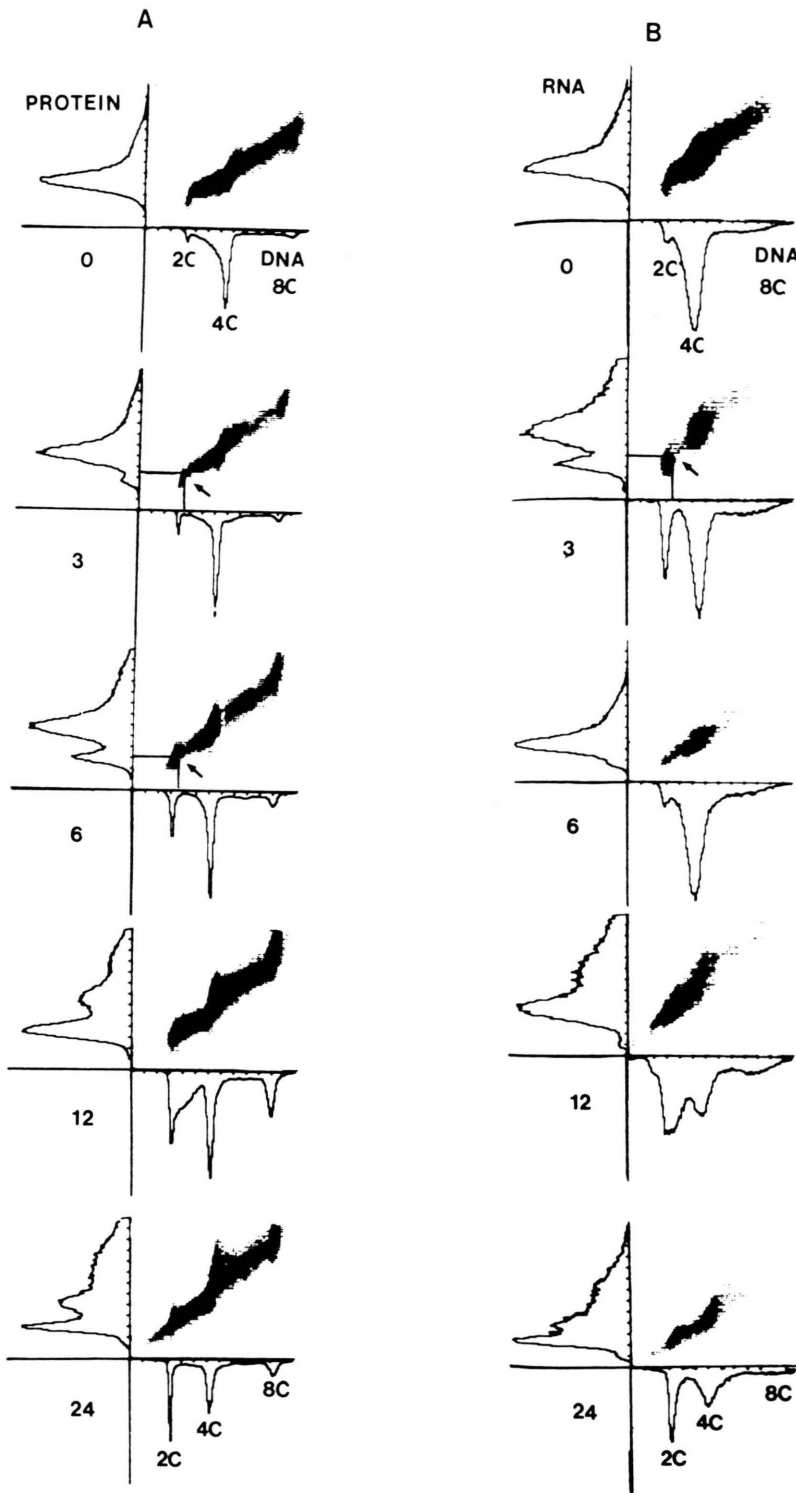
Fig. 7. Dual-parameter flow cytometry of G2 enriched populations of anaerobically treated EAT cells. A) DNA- and protein profiles and scattergrams. DNA staining with H33258, protein staining with sulforhodamin 101. B) DNA- and RNA profiles and scattergrams; staining of DNA and RNA with acridine orange. The arrows indicate the minimal protein (RNA) content of the S cells of the 2C → 4C cycle.

content is produced by hypoxia as we have proposed in a previous paper [19] and has been assumed by Shrieve *et al.* [31]. Recently, it was demonstrated by Schimke *et al.* [35] that transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification (over-replication) in Chinese hamster ovary cells. Similar effects have been seen for other DNA synthesis inhibiting agents. Inhibition of DNA synthesis followed by a period of release from the inhibitor may increase the frequency of gene amplification [32]. Irradiation [33] and Nocodazol [34] are further factors which may provoke polyploidization.

It is quite evident that the study of proliferation kinetics and biochemical parameters of cells reaerated after a period of anoxia has many practical and theoretical aspects. Cancer research and therapy, mechanisms of polyploidization, gene regulation and the toxicity of oxygen are only a few of these aspects [36].

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