

Growth Characteristics of Anaerobically Treated Ehrlich Ascites Tumor Cells after Reaeration as Studied by Combination of Flow Cytometry and Centrifugal Elutriation

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Centrifugal elutriation was applied to separate into the cell compartments asynchronous Ehrlich ascites cells grown under different culture conditions. The cytokinetic properties of the recultivated fractions were studied by flow cytometry. The present experiments prove that G1-cohorts grown 12 h under exclusion of oxygen accumulate in the late G1-compartment. The cytokinetic properties of these cells are not changed. In contrast the cytokinetic properties of anaerobically treated S-phase cells are changed; most of S-cells leave the cell cycle after they have attained the DNA content of G2-cells and continue to synthesize DNA without preceding division. Cells with a DNA content up to the fourfold of normal values are found.

Studies of the effects of deprivation of oxygen on the proliferation and the cell cycle of mammalian cells *in vitro* have shown that long before anaerobically grown cells die and disintegrate, they lose their capacity for proliferation [1–6]. Although the role that oxygen plays in regulating normal cellular metabolism is not well understood, it is quite clear, that exclusion of oxygen results in a series of physiological and biochemical changes, which include a complete inhibition of respiratory chain and oxidative phosphorylation as well as of all oxygen dependent metabolic processes and an increased rate of glycolysis [7] and adenine nucleotide degradation [8].

In our previous studies [2, 5, 6, 9] on the effects of oxygen deprivation on cell growth, cell cycle and metabolism of Ehrlich ascites tumor cells we have shown that 6–8 h after establishment of anaerobiosis the cells lose their capacity for proliferation and accumulate in the late G1-phase; during this time of anaerobiosis late S-cells enter the G2-compartment but do not divide, while cells which are in G2 + M at the beginning of exclusion of oxygen divide and enter G1. Our experiments revealed further, that S-phase cells are most sensitive to deprivation of oxygen. In an effort to characterize further the cytokinetics of anaerobically treated Ehrlich ascites tumor cells after reaeration we have applied a combination of centrifugal elutriation and

flow cytometry. In the present report we describe the results of this approach.

Materials and Methods

Chemicals, buffer and media substances were of the purest grade available from Merck, Darmstadt; Serva, Heidelberg and Sigma, München. Ethidium-bromide was from Serva. Argon/CO₂ = 95:5 and air/CO₂ = 95:5 were obtained from Messer-Griesheim, Frankfurt. Microcillin was a gift from Bayer, Leverkusen, horse serum was a gift from Behringwerke AG, Marburg. Oxsorb-catalyst R3-11/M 3610 was from BASF, Ludwigshafen.

Cell line and culture technique

Hyperdiploid Ehrlich ascites tumor cells, strain Karzel, were used for all experiments. Further details on culture conditions, flow cytometric analysis, and analytical procedures are described in loc. cit. 6.

Separation of cell compartments by centrifugal elutriation

Cells were fractionated using a Beckman IE-6-IM-2-elutriator rotor driven by a Beckman I2-21 centrifuge. The centrifuge speed was controlled with a ten turn potentiometer to permit rotor speed selection to within ± 10 revolutions per minute. Fluid flow through the elutriator system was maintained by a

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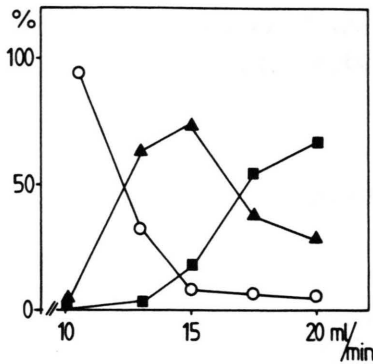


Fig. 1. Relationship between medium flow rate and phase composition of the fractions isolated from *in vivo* grown ascites tumor cells by centrifugal elutriation. Populations with > 90% G1-phase cells of about 75% S-phase cells and 66% G2 + M-phase cells were obtained in this experiment. ●—● G1-phase, ▲—▲ S-phase ■—■ (G2 + M)-phase cells. The yields of the cell compartments depend upon the phase composition at the beginning of the separation procedure.

Masterflex pump with fine velocity control. Prior to elutriation, the rotor was checked for possible external or internal leakage through defective seals or O-rings.

In a typical experiment the separation chamber was loaded at a rotor speed of 1580 rpm and a medium flow rate of 9 ml/min. Elutriation of different sized cells was performed by increasing the medium flow rate at a constant rotor speed. The relationship between flow rate and phase composition is demonstrated in Fig. 1. This diagram represents the results of a typical separation experiment of an *in vivo* grown ascites tumor harvested 5 days after inoculation.

Further details are described in reference [10].

Results

Cytokinetic properties of anaerobically treated G1-phase cells after reaeration

Applying centrifugal elutriation it was possible to obtain G1-phase populations of EAT cells with 95% homogeneity as checked by flow cytometry. Separation experiments were performed with a) cells harvested from the peritoneum of mice, b) cells cultured 24 h *in vitro* and c) 12 h anaerobically treated cells. The proliferation kinetics of the recultivated G1-cells over a period of 24 h are depicted in Fig. 2.

From this figure we may conclude that anaerobically treated cells have passed the G1-period about 9 ± 1 h after recultivation; 8 h after beginning of the experiment most of these cells have entered the S-phase, they traverse the cell cycle synchronously up to about 16 h, thereafter desynchronization begins. 24 h after reaeration this population contains about 30% G1-, 40% S- and 30% G2 + M-phase cells. At the same time when anaerobically cultured cells have completely left the G1-compartment, 30% of the G1-cells isolated from the peritoneum of mice, and 50% of the G1-cells isolated from aerobically grown cultures are still present in the G1 compartment. These data prove our previous observation [5, 6] that anaerobically cultured cells accumulate in the late G1-period. The G1-period of *in vitro* grown cells generally lasts 18 ± 1 h. The cytokinetic properties of 12 h anaerobically treated G1-cells are not changed by a limited exclusion of oxygen.

A comparison of the proliferation kinetics of 12 h anaerobically grown G1-cells, as well as of the increase of total cell number and of the protein/ 10^6 cells is given in Fig. 3. The data demonstrate that 16 h after recultivation total cell number begins to increase. This time period corresponds to the dura-

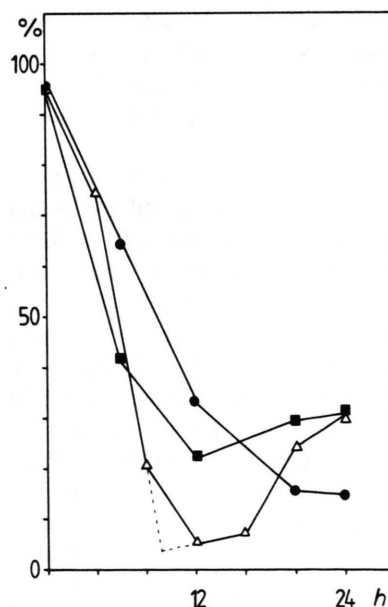


Fig. 2. Proliferation kinetics of recultivated G1-phase cells obtained by centrifugal elutriation from a) ■—■ cells harvested from the peritoneum of mice; b) ●—● aerobically grown cells *in vitro*; c) △—△ 12 h anaerobically treated cells.

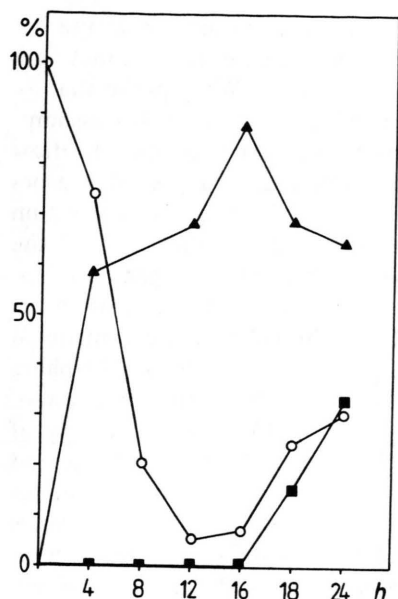


Fig. 3. Comparison of cell growth ■—■, protein/ 10^6 cells ▲—▲ and kinetics of the G1-period ○—○ of anaerobically treated G1-cells.

tion of the S + G2 + M phase; at the same point the G1-cells traverse a minimum and the protein content per 10^6 cells decreases, because cells begin to divide. A satisfactory correlation between flow cytometric data and the other parameters of cell proliferation is demonstrated by this diagram.

The effects of deprivation of oxygen on the proliferation kinetics of anaerobically treated S-phase cells after reaeration

With the exception of G1-cells, synchronous populations of S- and G2-cells separated by centri-

fugal elutriation are generally < 80% pure [11]. In the present experiments we obtained fractions with about 70–80% S-phase cells. These fractions were well suited to study the cytokinetic properties of S-cells after a 12 h period of deprivation of oxygen. DNA-histograms of recultivated S-cells isolated from anaerobically treated populations and controls are depicted in Fig. 4. While DNA histograms of control cultures show a normal cell cycle progression, histograms of 12 h anaerobically treated cells reveal significant disturbances of the growth pattern which are characterized by the appearance of fluorescence signals on the right of the G2 + M-peak and of a peak corresponding to cells with a DNA content of 8c. These unexpected signals indicate that a great part of S-phase cells do not divide, they rather leave the cell cycle and continue to synthesize DNA after they passed the G2-compartment. We suggest that especially late S-phase cells are changed by deprivation of oxygen; obviously they have lost the ability to enter mitosis (see below). The kinetics of cell cycle progression of the S-phase cells is shown in Fig. 5. About 8 ± 1 h after recultivation, the S-cohorts pass a minimum, at the same time the G2 + M populations reach a maximum (not shown). This time period approximately corresponds to the duration of the S-phase of the Ehrlich ascites cells grown under our conditions.

In order to characterize further the cytokinetic properties of the late S-phase cells, populations with a high content of late S-phase and G2 + M-phase cells which cannot be separated by centrifugal elutriation, were recultivated. DNA-histograms of these cells are shown in Fig. 6.

These histograms confirm that part of the S-cells of anaerobically treated cultures after reaeration

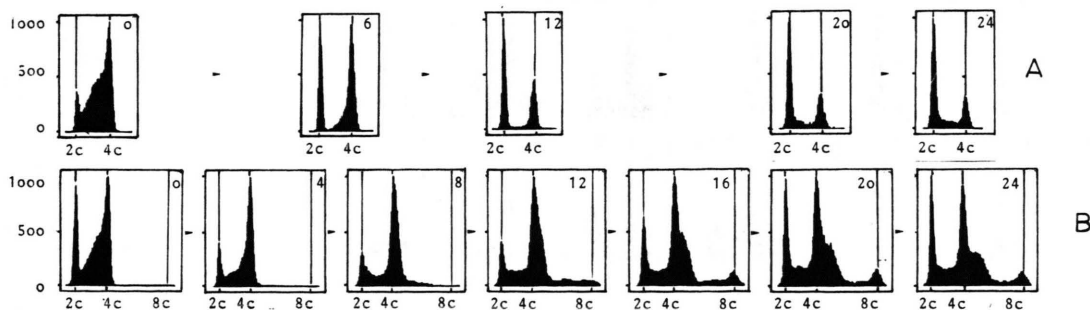


Fig. 4. DNA-histograms of S-phase cells enriched by centrifugal elutriation. (A) separated from aerobically cultured controls (G1 = 5%, S = 75%, G2 + M = 19%) (B) isolated from 12 h anaerobically treated cultures (G1 = 9%, S = 72%, G2 + M = 19%).

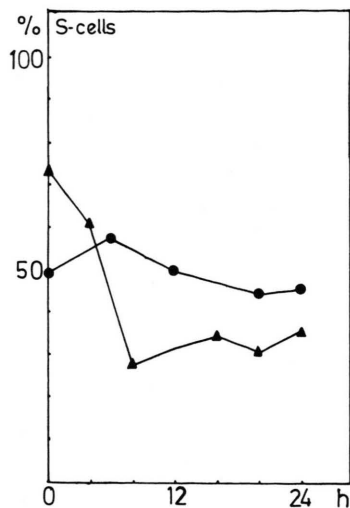


Fig. 5. Proliferation kinetics of recultivated S-phase cells separated by centrifugal elutriation from a) aerobically grown control cultures ●—●; b) 12 h anaerobically grown cells ▲—▲.

leave the cell cycle and begin again to synthesize DNA without cell division. Populations which at the beginning of recultivation do not contain polyploid cells (DNA-histograms series B), reveal fluorescence signals above 4c already 8 h after reaeration. These signals increase rapidly and after 16 h a peak has developed corresponding to cells with a DNA content of 8c. Further informations are obtained from the histogram matrix depicted in

Fig. 6c. Tetraploid cells present already at the beginning of recultivation increase up to about 16 h and thereafter decrease again. We suppose that 8c cells pass a second cell division cycle; this assumption is checked by further experiments. The flow cytometric histograms of Fig. 6 suggest, that especially late S-phase cells are impaired by deprivation of oxygen. There are further indications of the destabilizing effects of exclusion of oxygen on the S-phase cells coming from the growth curve of anaerobically treated S-cells fractionated by centrifugal elutriation. In Fig. 7 growth curves of G1-phase cells and of populations with a high fraction of S-phase cells are depicted. 16 h after beginning of recultivation the G1-phase cells start to divide and cell number increases; this time period corresponds to the length of S + G2 + M. Fig. 7 demonstrates further that populations with a high fraction of S-phase cells decrease by about 20% during the first 4 h after reaeration. Only additional 12 h later cell number increases. Obviously late S-phase cells do not only not divide but a considerable quantity of them dies after restoration of aerobic conditions. This seems to be the reason why the cell number of anaerobically treated, asynchronous cultures does not increase before 12 h after reaeration, although late S-phase and G2 + M-phase cells are present at the end of anaerobiosis and the duration of the G2 + M period is about 8 ± 1 h.

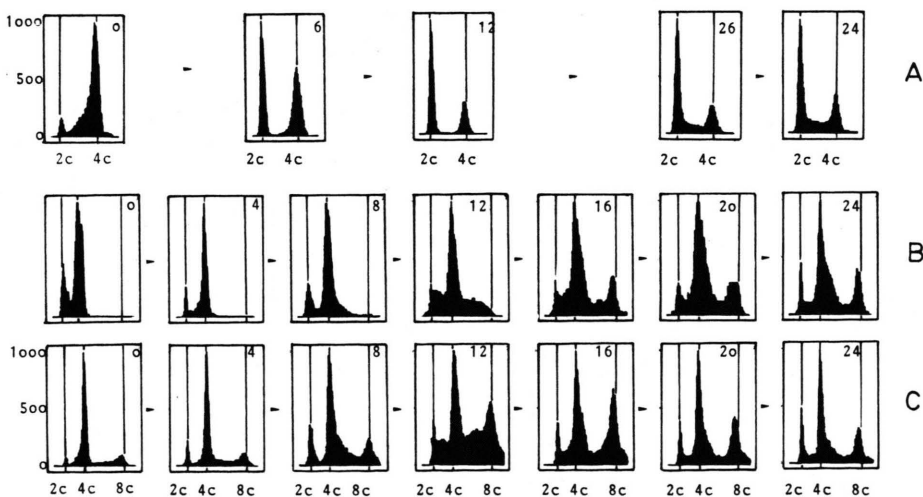


Fig. 6. DNA-histograms of cell populations containing more than 85% of late S-phase and G2 + M-phase cells. The fractions were obtained by centrifugal elutriation from (A) Ehrlich ascites cells harvested from the peritoneum of mice (B) 12 h anaerobically treated cells, (C) 12 h anaerobically treated cells. This fraction contained tetraploid cells (DNA = 8c) from the beginning of recultivation.

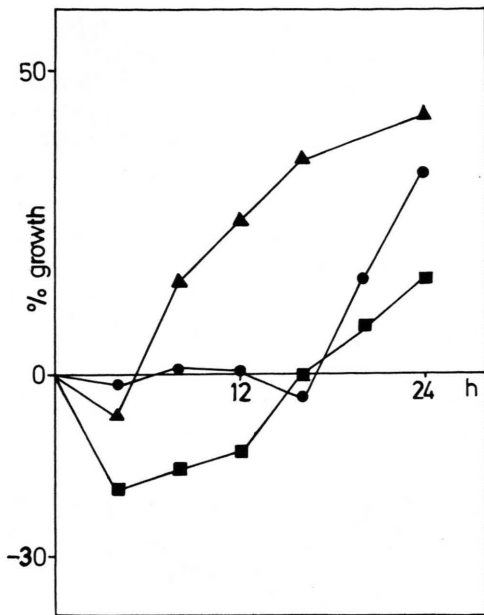


Fig. 7. Growth of recultivated cell fractions isolated from anaerobically treated populations by centrifugal elutriation. ●—● pure G1-fraction. ■—■ fraction containing 40% G1, 46% S and 2.2% G2 + M cells, ▲—▲ fraction containing cells with a DNA content > 4c.

Discussion

A combination of centrifugal elutriation and flow cytometry as applied in the present experiments allows a subtle analysis of the effects of exclusion of oxygen on the proliferation kinetics of anaerobically cultured Ehrlich ascites tumor cells after reoxygenation. This approach offered the possibility to supplement and to extend our previous investigations on the role of oxygen in the cell cycle of EAT cells [5, 6, 10].

It was possible by centrifugal elutriation to attain G1-fractions of > 95% homogeneity as analyzed by flow cytometry, though the G1-fractions isolated from anaerobically treated populations preferentially contain late G1-cells which are very similar to early S-phase cells with respect to size and density. While in the present experiments we succeeded to obtain S-phase cells with a purity of 70–80%, the separation of late S and early G2 + M-cells was more problematic. Fractions containing about 50% S- and 50% G2 + M-cells could be obtained from cell populations cultured *in vivo* in the peritoneum of mice. It may be possible to improve these results

by starting the separation procedure with cell populations containing higher fractions of G2 + M-phase cells.

The observation that a 12 h exclusion of oxygen causes an accumulation of the cells in the late G1-period prompts the question how the transition of the cells from G1 to the S-phase is controlled by oxygen. We presently do not assume, that the arrest of cell cycle progression after deprivation of oxygen in late G1 compartment is related to restriction or commitment point at which a cell decides whether to initiate DNA synthesis and undergo division or to cease proliferation [12]. It is well known that many tumor cells and transformed cells have lost the commitment point control in whole or in part. We have further observed [6] that about 20 h after establishment of exclusion of oxygen cells begin to leave the G1 compartment and to enter the S-phase. It may be possible that essential components, which cannot be synthesized in the absence of oxygen, are made available from disintegrated cells and promote cell proliferation after they have accumulated. This hypothesis is under investigation in our laboratory.

In previous studies, we have already shown that growth inhibition by deprivation of oxygen is reversible upon reexposure of the cultures to oxygen [5, 6]. It may therefore be possible to use exclusion of oxygen to obtain a reversibly inhibited G1-enriched population. These semisynchronous populations are well suited for the isolation by centrifugal elutriation of G1-fractions of high purity in high yields; a limited deprivation of oxygen obviously does not impair the cells present in the G1 compartment; their cytokinetic properties are not changed by an 12 h exclusion of oxygen. The relation between the duration of exclusion of oxygen on one side and the changes in cellular economy and proliferation kinetics on the other side requires further investigations.

As shown in previous experiments, late S-cells enter the G2-phase during the first 6–8 h of anaerobiosis [6]; these G2-cells do not leave the compartment before reoxygenation. Most S-cells remaining in their compartment during exclusion of oxygen are damaged. After reoxygenation they do not enter mitosis but continue to synthesize DNA without division; the DNA content of these cells increases up to 8c. The molecular mechanisms underlying the loss of control of DNA synthesis and cell division of anaerobically treated cells after reoxygenation

tion are not well understood. Damage to DNA is reported to prevent progress towards mitosis [13]. It is further known that polyploid cells may arise in the presence of several drugs (colcemid) or by changing environmental conditions [14]. Impairment of the synthesis of the mitotic apparatus or resorption of its components under anaerobic conditions must further be taken into consideration. Deprivation of oxygen indeed reduces protein synthesis of the cells to about 40% of aerobic controls [2], but this seems not be the decisive point, since transformed cells continue to cycle until protein synthesis is 90% inhibited, whereas normal cells become

arrested already at 65% inhibition [15, 16]. Exclusion of oxygen may be considered as a sort of nutrient deficiency; depletion of nutrients, for example amino acids, is known to arrest some chemically transformed cells in the G1 stage [7]. Preliminary experiments with EAT cells cultured in media depleted of single amino acids however have shown a completely different pattern of proliferation than anaerobically grown cells. The molecular mechanisms underlying the growth arrest of anaerobically grown mammalian cells remain to be clarified.

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