Growth Characteristics of Anaerobically Treated Early and Late S-Period of Ehrlich Ascites Tumor Cells after Reaeration

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Dedicated to Prof. E. Buddecke *on the Occasion of His 60th Birthday*

Proliferation Kinetics, Ehrlich Ascites Cells, Anaerobiosis

Utilizing centrifugal elutriation, early and late S-phase cells were separated from 4, 8 and 12 h anaerobically cultured Ehrlich Ascites tumor cells strain Karzel. The cytokinetic properties of these fractions after reaeration were studied by flow cytometry and the BrdU-H 33258-technique of flow cytometry. After a 4 h period of anaerobiosis, growth of early S-phase cells is not changed, 8 h deprivation of oxygen causes a delay of cell cycle progression, while the main fraction of 12 h anaerobically treated early S-populations did not divide after reaeration within 24 h. In comparison to early S-phase cells the cell cycle progression of the main fraction of late S-period is accelerated after a 4 h exclusion of oxygen. A fraction of 8 h anaerobically pretreated late S-cells continues to cycle, but a considerable number reinitiates DNA synthesis without preceeding division. Cells with DNA content up to 8 c are detected by flow cytometry. 12 h anaerobically cultured late S-cells do not divide after reaeration, a large number of these cells starts again to synthesize DNA. A considerable part of tetraploid cells retain viability, divide and enter a new cell cycle, another part of the cells disintegrates.

Previous investigations on the effects of deprivation of oxygen on the proliferation kinetics of *in vitro* cultured asynchronous EAT cells after reaeration have shown that S-phase cells are most sensitive to exclusion of oxygen [1–4]. Their cytokinetic properties after reaeration are significantly changed, the control of DNA synthesis is severely impaired and a significant quantity of the S-cells die after recultivation under aerobic conditions.

Using centrifugal elutriation we have isolated from anaerobically treated asynchronous cultures fractions with a high content of early S-cells and other fractions with a high content of late S-cells. Flow cytometric techniques have been utilized to study the proliferation kinetics of these fractions after establishing normal culture conditions. In the present communication we report the results of these experiments, which have shown that deprivation of oxygen induces different changes of growth characteristics depending on the time of oxygen exclusion during the cell cycle and that those S-cells, which leave the cell cycle after reaeration and continue to synthesize DNA without preceeding division, enter a second division cycle after they have attained a DNA content of tetraploid cells.

Abbreviations: EAT-cells, Ehrlich Ascites Tumor cells; ETB-histograms, DNA-histograms of cells stained with ethidium bromide; BrdU-histograms, DNA-histograms of cells grown in the presence of 5-bromo-deoxyuridine and stained with benzimid H 33258

Reprint requests to Prof. Dr. Fr. Schneider.

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Materials and Methods

Chemicals

All chemicals, buffer and media substances were of the purest grade available from Merck (Darmstadt), Serva (Heidelberg), and Sigma (München). Ethidiumbromide, BrdU were from Serva, Benzimid H 33258 was from Riedel de Haen (Hannover). Argon/CO_2 = 95:5 and air/CO_2 = 95:5 were obtained from Messer-Griesheim.

Cell strain and growth conditions

A strain of hyperdiploid Ehrlich ascites tumor (EAT) cells subcultured by Karzel [5] was used for the experiments. Details of cell culture procedure are described in [2] and [3].

Flow cytometry

Sequences of DNA histograms were used for monitoring the cell cycle distribution of the cell cultures. Fractions of cells in G1-, S- and G2M-phases of asynchronous cells were evaluated from the histograms assuming Gaussian distributions under the

G1 and the G2M peak and attributing the rest of the cells to the S-phase. Ethidiumbromide staining of the cells was combined with the BrdU-H 33258 technique [6, 7] for flow cytometrical cell cycle analysis. This latter technique uses the quenching of H 33258 fluorescence by BrdU substitution for thymidine, thus leaving every cell in its fluorescence compartment during further progression through DNA synthesis until division. For further details see 2, 3 and 4.

Fractionating of asynchronous cultures by centrifugal elutriation

Fractions with a high content of early and late S-phase cells were obtained using a Beckman JE-6-JM-2 elutriator rotor driven by a Beckmann J2-21 centrifuge as described in loc. cit. [2] and [4]. 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 Masterflex pump with fine velocity control. In a typical experiment, the separation chamber was loaded with about 2×10^8 cells (8×50 ml standard cultures) at a rotor speed of 1700 rpm. Elutriation of "early" S-phase cells was performed at a flow rate of 15–18 ml/min, of "late" S-cells at a flow rate of 20–25 ml/min.

Results

EAT cells harvested from the peritoneal cavity of female NMRJ mice 5–7 days after inoculation were cultured anaerobically for 4, 8 and 12 h after a first *in vitro* passage of 13–15 h under standard conditions. The anaerobically grown populations were separated by centrifugal elutriation. As judged by flow cytometrical analyses and the BrdU-H 33258 technique of flow cytometry it was possible to obtain fractions with about 50% S-phase cells. These fractions could be subdivided into early S-cells which contained about 50% late G1-cells and into late S-cells containing about 50% G2-cells. These subfractions were recultivated under aerobic conditions in order to obtain further informations on the effects of deprivation of oxygen on the proliferation kinetics of different stages of the S-compartment after reaeration.

The effects of a 4 h period of anaerobiosis

After a 4 h period of exclusion of oxygen, the growth of recultivated early S-Phase cells is not significantly impaired as is demonstrated in Fig. 1. 12 h after reaeration, no signals of cells with a DNA content $>2c$ can be detected in the BrdU-histogram (Fig. 1 B), which indicates, that the S-cells of the population have divided, their fluorescence signals are now detectable between 1c and 2c peak. The 2c peak (G2-cells) of the BrdU-histogram after 12 h recultivation stems from G1-cells which have not yet divided at this time. The fluorescence signals of these cells correspond to the 4c peak of the ETB-histogram (Fig. 1 A/12 h). 24 h after reaeration nearly the whole polulation has passed mitosis.

From the DNA histograms of Fig. 2 we may conclude that the main fraction of 4 h anaerobically treated late S-Phase cells has divided 12 h after reaeration. Some signals can be detected between 2c and 4c in the 12 h and 24 h BrdU-histograms, indicating that a small fraction of 4 h anaerobically treated late S-phase cells do not divide within 24 h. The broad 2c peak of the 12 h BrdU-histogram (Fig. 2 B, 12 h) corresponds to fluorescence signals of G2- and late S-cells after division. 24 h after beginning of aerobic recultivation the main part of the cells has already entered the next cell cycle as may be reduced from the appearance of an 1c (G1/2) peak and from the signals between 1c and 2c (Fig. 2 B, 24 h). Since at the beginning of recultivation these cell cultures really do not contain G1-phase cells, an 1c (G1/2) peak can only arise after

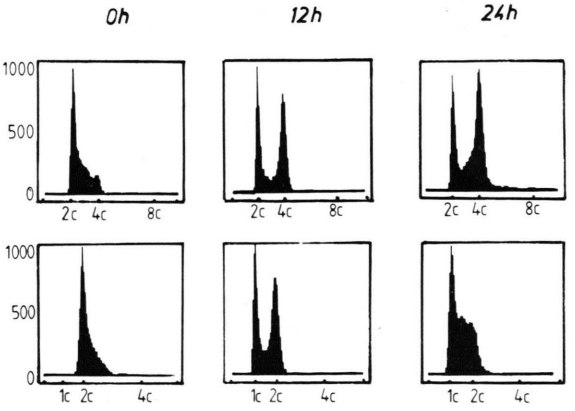

Fig. 1. (A) ETB-histograms of 4 h anaerobically treated early S cells after reaeration. (B) BrdU-histograms of the same cells. Abscissae: DNA content (fluorescence intensity). Ordinates: Counts per channel (cell number).

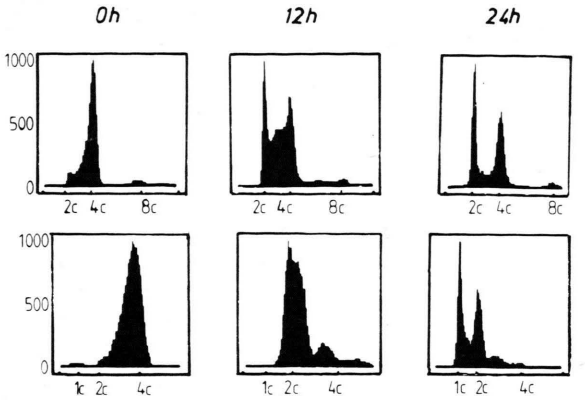

Fig. 2. (A) ETB-histograms of 4 h anaerobically treated late S cells after reaeration. (B) BrdU-histograms of the same cells.

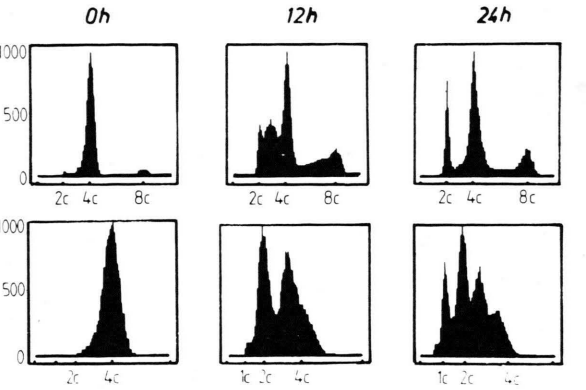

Fig. 4. (A) ETB-histograms of 8 h anaerobically treated late S cells after reaeration. (B) BrdU-histograms of the same cells.

the cells have passed the following cell cycle. We therefore must infer that the main fraction of 4 h anaerobically treated late S cells traverse the cell cycle within less than 20 h. The cell cylce time of this cell strain in the first and second in vitro passage under normal conditions is more than 30 h. A short time exclusion of oxygen obviously speeds up the cell cycle progression of this cell fraction after reaeration.

The effects of an 8 h period of anaerobiosis

Histograms of 8 h anaerobically treated early S-phase cells after reaeration over 24 h are depicted in Fig. 3. These histograms reveal that 8 h after exclusion of oxygen, early S-cells traverse the cell cycle more slowly than after 4 h of anaerobiosis. This becomes evident from a comparsion, of the histogram series 1 B and 3 B. 12 h after reaeration, most of the BrdU labelled cells have not left their fluorescence compartment indicating that they did not divide. These statements are confirmed by the ETB-histograms: 8 h anaerobically treated cells have attained about 10 h later the same cell cycle distribution as 4 h anaerobically cultured early S-phase-cells (compare Fig. 1 A, 12 h- and Fig. 3 A 24 h-histograms).

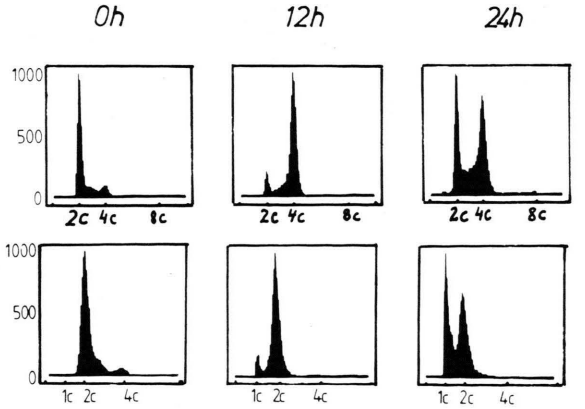

Fig. 3. (A) ETB-histograms of 8 h anaerobically treated early S cells after reaeration. (B) BrdU-histograms of the same cells.

Histograms of 8 h anaerobically cultured late S-cells after reaeration are demonstrated in Fig. 4. This period of exclusion of oxygen has distinct effects on the cytokinetics of late S-cells after reaeration. While a smaller number of these cells passes the cell division cycle with some delay, another fraction continues to synthesize DNA after the cells have attained the DNA-content of G2 and fluorescence signals of cells with a DNA content > 4c are detected in both histogram series. 12 h after starting reculti-vation an 8c peak is observed corresponding to tetraploid cells (Fig. 4 A, 12 h). A further peculiarity of the BrdU-histograms is the appearance of a peak between 2c and 4c (Fig. 4 B, 24 h). At the present time we suggest that these signals stem from the division of 8c cells. These are the first indications that 8c cells divide again and that cells with an abnormal DNA content arise. This problem is further discussed below.

The effects of a 12 h period of anaerobiosis

BrdU-Histograms of 12 h anaerobically treated early S-phase cells (Fig. 5) reveal that the late G1 cells of these populations have not divided after 12 h

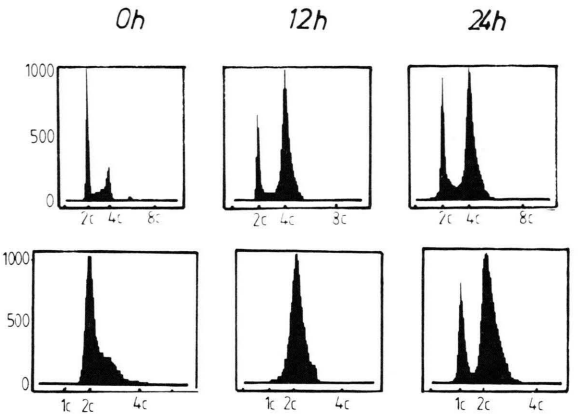

Fig. 5. (A) ETB-histograms of 12 h anaerobically treated early S cells after reaeration. (B) BrdU-histograms of the same cells.

(no fluorescence signals at G1/2 = 1c), only 12 h later an 1c peak appears. Cell cycle progression of the G1 fraction is delayed; a considerable quantity of the S-cells do not divide; some of the cells continue to synthesize DNA without preceeding division: Fluorescence signals of cells with a DNA content > 4c appear, which becomes evident from a shoulder at the right of the 4c peak and broadening of this peak in the 24 h ETB-histogram (Fig. 5A). The small peak near 4c in the ETB histogram (Oh) stems not from G2-cells but are signals from aggregated G1 cells [8].

The effects of an 8 and 12 h deprivation of oxygen on the proliferation kinetics of late S-phase cells after recultivation under aerobic conditions are similar (Fig. 6). Most of the late S- and G2-cells do not enter mitosis and do not divide: 12 h after reaeration a significant number of cells with an abnormal high DNA content can be detected by flow cytometry (Fig. 6A, 12 h). Further 12 h later a peak of fluorescence signals corresponding to cells with a DNA content of 8c appears (Fig. 6A, 24 h). Within 24 h after beginning of aerobic recultivation most of late S-phase cells have started to synthesize DNA again after they have attained the DNA content of G2 cells. A minor fraction of these cells are polyploid after this time period (Fig. 6A, 24 h). Further experiments support our assumption that 8c-cells enter a new cell cycle and divide again.

Tetraploid cells enter a new cell division cycle

Applying centrifugal elutriation, fractions with a significant number of tetraploid cells were isolated and recultivated under aerobic conditions. Autoradiographic studies with these cells have shown that after 28 h incubation with [^{3}H]thymidine, practically all cells are labelled, which implicates that they have entered the S-phase and synthesized DNA [4]. DNA-histograms of a recultivated fraction consisting of about 75% G2-, 16% S- and 9.0% G2* (8c-)cells are depicted in Fig. 7. From these histograms the following conclusions may be drawn: 6 h after recultivation part of the G2 cells have divided and a rather symmetrical G1 peak is obtained. Within the first 6 h, the main fraction of G2 cells has however started to synthesize DNA again without preceeding division and a great number of cells with a DNA content between 4c and 8c has formed. Cell cycle distribution at this time is about 15% G1-, 3% S-, 29% G2-, 43% S*-, 10% G2* (8c-)cells. The 12 h histogram shows that most of G1 cells have entered

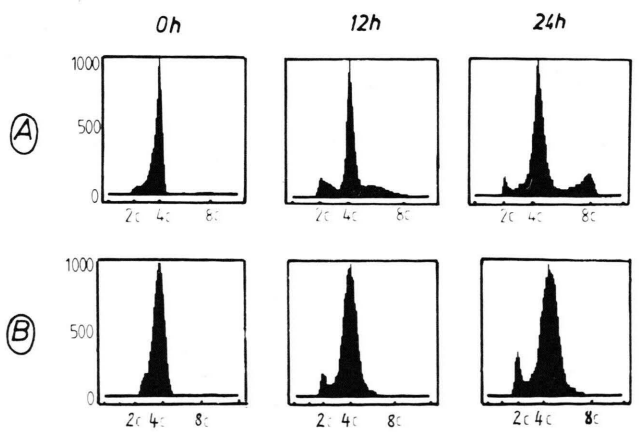

Fig. 6. (A) ETB-histograms of 12 h anaerobically treated late S cells after reaeration. (B) BrdU-histograms of the same cells.

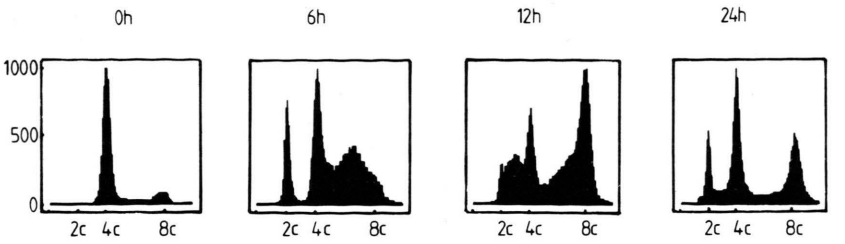

Fig. 7. ETB-histograms of a recultivated elutriator fraction containing 75% G2 and 9% 8c-cells. The fraction was separated from 8 h anaerobically treated cultures.

the S-period and that the cells with a DNA content beyond 4c move into the 8c compartment; apparently they travers a following cell division cycle. 24 h after beginning of recultivation 8c cells have divided to form 4c cells. "Normal" cells have passed the cell cycle after this time period, which may be concluded from an increase of the G1 (2c) peak. It seems evident that the histogram series is the result of two parallel proceeding cell division cycles. The 4c peak corresponds to fluorescence signals of G2M cells of the first division cycle as well as of G1 cells of a second cycle. The cell cycle distribution is now about 12% G1-, 11% S-, 31% G2M + G1*-, 18% S*- and 28% G2*-cells. The histograms also demonstrate that some of the G2 cells do not leave their compartment or enter mitosis only very slowly after reaeration and that the cell cycle time of the first cycle is about 10 h.

Concluding remarks

Under prolonged hypoxia profound changes in cell proliferation of ETA cells occur within a few hours. Previous investigations 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 [3, 4]; they accumulate in the late G1 period but do not enter the S-phase under these conditions. After reaeration the cell cycle progression of anaerobically treated G1 cells may be delayed depending on the time of exclusion of oxygen. 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.

As is shown in the present paper a more complex response to exclusion of oxygen of S-phase cells becomes evident after reaeration. From our results we must conclude that the effects of anaerobiosis on S cells depend on the stage where the cells are within this compartment (for instance early or late) and the time period of anaerobiosis during the cell cycle. The most prominent observation is that preferentially late S-phase cells leave the normal cell cycle and continue to synthesize DNA without preceeding division after they have attained the DNA content of G2 cells. They achieve the DNA content of tetraploid cells and presumably divide again. Some of the late S cells die [4]. The acceleration of cell cycle progression of short time anaerobically treated late S cells after reaeration is probably due to a reduced G1 period. Further experiments are under way to clarify this question. S cells which have entered G2 during anaerobiosis show similar proliferation characteristics after reaeration as late S cells which remained in their compartment.

The biochemical signals for onset and cessation of DNA synthesis are still elusive; therefore the mechanisms underlying the impairment of S cells by exclusion of oxygen are not yet understood. Concerning the most important event of the S period, namely DNA synthesis it was shown by Probst *et al.* [9] that deprivation of oxygen causes a rapid and reversible inhibition of replicon initiation of DNA synthesis of Ehrlich ascites tumor cells; in reaerated cells, the initiation pattern of the daugther chains of operating units was normal. The molecular basis of the oxygen dependence of the replicon initiation is not clear. Our results presented in [1, 10, 11] indicate that glycolysis can supply sufficient ATP and that energy deficiency may not be the reason. Studies on the role of dihydro-orotate oxidase in the inhibition of DNA synthesis of Ehrlich ascites tumor cells cultured under exclusion of oxygen have shown [12] that DNA synthesis may be arrested only by inhibition of this enzyme of pyrimidine biosynthesis, which is linked to the electron transport chain and cannot operate under anaerobic conditions.

It is further suggested, that dCTP and dTTP play a regulatory role in the biosynthesis of DNA. dCTP is a precursor, the level of which acts as a threshold for

the onset of DNA synthesis; dTTP, also a precursor, can inhibit the conversion of CDP to dCTP and the deamination of dCMP [13]. Experiments in our lab have shown indeed, that cells arrested in late G1 under exclusion of oxygen enter the S phase after supplementation with deoxycyctidine [14].

Several other experiments indicate the existence of some essential contributions of the S-phase cytoplasma to the regulation and maintainance of DNA synthesis. It is assumed that an inducer of DNA synthesis is produced at the beginning of the S-phase and probably is maintained throughout the S-period. The inducer is apparently absent during the rest of the cell cycle. Obviously in anaerobically treated late S-cells the DNA remains responsive to the inducer after cells have attained the DNA content of the G2 phase. Normally DNA in the G2-nucleus is in a state that cannot react to the inducing agent [15].

Experiments with different cell types have revealed that S cells generally are most sensitive not only to deprivation of oxygen but also to elevation of oxygen tension and to radiation. On exclusion of oxygen the cell cycle time of Chinese Hamster cells increases about five fold on account of a four to five fold increase in the duration of the S phase [16]. Cristofalo and co-workers have shown that initiation of DNA synthesis of diploid WJ cells is inhibited by an oxygen tension of $590 + 35$ mm Hg [17]. The same authors have observed a decrease of growth rate to $20-30\%$ of the controls at pO_2 of 29 mm Hg with these cells [18]. As was described by Beck [19], [^{3}H]thymidine labelled fibroblasts strain 4929 are delayed in their progression through the S and G2M phase; the results illustrate the very similar effects of deprivation of oxygen and of the radiation of incorporated [^{3}H]thymidine. Radiation induced progression delay in S phase and G2 block, depending on time of X-ray irradation in the cell cycle were also measured in synchronized Ehrlich ascites tumor cells [20]. However it should be emphasized that slowing down of the rate of DNA synthesis is itself not necessarily noxious to cells nor does a prolongation of the G1 phase damage cells.

Acknowledgements

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