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Centrifugal elutriation of hepatocytes from 2-acetylaminofluorene-treated rats and their characterization by flow cytometry

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Treatment of male Wistar rats with 2-acetylaminofluorene (2-AAF) markedly altered the ploidy distribution of liver cells. Small diploid hepatocytes first appeared after 4–5 weeks feeding of a diet containing 0.02% 2-AAF; after 9 weeks 65–70% of the hepatocytes were diploid. Approximately two-thirds of this new liver cell population persisted after termination of the treatment. The hepatocytes from 2-AAF treated animals were separated according to size and ploidy by centrifugal elutriation and stained for γ-glutamyltranspeptidase (γ-GTase). The percentage of γ-GTase-positive hepatocytes did not significantly differ between the various elutriated cell fractions. Thus γ-GTase-positive liver cells obtained by feeding of 2-AAF do not represent a distinct size class of hepatocytes. The significance of carcinogen-induced diploid hepatocytes in hepatocarcinogenesis is discussed.

Introduction

When administered to rats for a few weeks, several DNA-reactive carcinogens such as azo-compounds (1,2), aflatoxin B_1 (AFB_1*) (3) and diethylnitrosamine (DEN) (2) induce a marked decrease in the ratio of tetraploid/diploid hepatocytes in the liver. Even a single treatment with AFB_1 (3) or DEN after 2/3 partial hepatectomy (4,5) increases the percentage of diploid hepatocytes in rat liver. The emergence of small diploid hepatocytes can be markedly increased by sequential feeding of 0.02% 2-acetylaminofluorene (2-AAF) to 2/3 hepatotectomized rats treated with a single dose of DEN (4). It has been reported that tumours induced by the latter treatment, by AFB_1 or by feeding 0.025% 2-AAF followed by promotion with phenobarbital, are mainly diploid (3,4,6,7). Thus, under certain experimental conditions diploidization may represent an important step in hepatocarcinogenesis in the rat.

There is still little information on the process of diploidization in rat liver after 2-AAF. In the present work we used flow cytometry to study the time dependence of the emergence of diploid hepatocytes and their persistence after termination of the carcinogen treatment. To characterize hepatocytes further from 2-AAF-treated animals we separated the cells into seven fractions according to their size and ploidy using centrifugal elutriation. The liver cells in the various fractions were stained for γ-glutamyltranspeptidase (γ-GTase), which is a marker frequently used to detect putatively preneoplastic hepatocytes in enzyme-altered foci (8,9).

Materials and methods

Animals

Male Wistar rats (6 weeks old) from the GSF breeding colonies were fed a standard pellet diet (Altromin, Lage, FRG) containing 0.02% 2-AAF for 9 weeks or the time indicated. Except for the experiments shown in Figures 2 and 3 animals were kept on the standard diet without 2-AAF for at least 3 weeks before being used for the preparation of isolated hepatocytes.

Isolation of hepatocytes

Rats were anesthetized with sodium phenobarbital (9 mg/100 g body wt), the abdominal cavity was opened and the liver perfused via the portal vein with 250 ml of Ca^{2+}-free modified Hank's solution containing 100 μM ethylene glycol-bis (β-aminoethylether) *N-N'* tetraacetic acid (EGTA). After canulation of the vessel the flow rate was increased from ~25 ml to 40 ml/min. Subsequently perfusion was continued with 250 ml of the same Ca^{2+}-free buffer without EGTA, followed by recirculation with 100 ml Dulbecco's modified minimal essential medium (DMEM) containing 1.8 mM $CaCl_2$ and 100 U/ml collagenase (type CLS II) (Worthington Biochemical Corp., Freehold, NJ). After 10–15 min the Glisson's capsula was opened, the cells gently dispersed in DMEM and filtered through an 80-μm nylon mesh filter. Finally the cells were washed three times at 30 × *g* for 45 s. Under these conditions a partial purification of the cell suspension from non-parenchymal cells is achieved. Viability of the cells was determined with 0.4% trypan blue; >80% of the hepatocytes excluded the dye. When hepatocytes were to be subjected to centrifuged elutriation a second enzymatic treatment with collagenase was introduced to decrease the number of cell aggregates. 130 × 10^6 cells were incubated at room temperature in an atmosphere of 95% O_2 and 5% CO_2 in 50 ml DMEM medium containing 100 μg/ml DNase I (bovine pancreas 3500 U/mg, Boehringer Mannheim, FRG) and 95 U/ml collagenase. After 30 min the cell suspension was filtered twice through a 40-μm nylon mesh. This treatment reduced the number of cell aggregates (doublets) from ~30 to 20% in hepatocyte suspensions obtained from 2-AAF-treated animals and decreased the percentage of cells which were stained by trypan blue to <3% (10).

Fixation, staining of DNA and detection of γ-GTase-positive hepatocytes

Cells suspended in PBS were fixed with an equal volume of methanol for 10 min at 4°C; subsequently they were washed with PBS and incubated in 2 ml PBS containing 200 μg RNase A (bovine pancreas 90 U/mg, Sigma, Deisenhofen, FRG) at 37°C for 30 min. Subsequently, the DNA was stained with 60 μM propidium iodide for 15 min.

γGTase was demonstrated in air-dried cell smears according to Lojda *et al.* (11). One thousand cells in 10–20 areas were counted per slide.

Flow cytometric analysis

Flow cytometry was carried out using a FACS-Analyser (Becton-Dickinson). In order to exclude non-parenchymal cells from the measurement, the instrument was adjusted so that only cells >15 μm were monitored. Volume was determined by electrical resistance pulse sizing (Coulter-sizing) using a 75- or 100-μm orifice. DNA was quantitated at excitation and emission wavelengths of 470–495 and 550–600 nm respectively; 10 000 events were counted routinely. Data analysis was performed using the Becton-Dickinson Consort 30 Program version E 12/86.

Calculation and correction of the data were performed as follows. In this study cellular ploidy has been studied, i.e. mono- and binucleated cells are not treated as separate cell populations. The four 'hepatocyte populations' which can be discriminated in the histogram (Figure 1), can be attributed to the following cellular ploidy classes: 2c, '4c', '6c' and '8c'. However, while 2c represents exclusively single diploid cells, it is necessary to correct the other ploidy classes for the presence of cell aggregates. '4c' represents either tetraploid cells or cell doublets of two diploid cells. '6c' only represents cell aggregates of a diploid and a tetraploid cell. '8c' represents octaploid cells and cell doublets consisting of two tetraploid cells. After determination of the number of cell aggregates in the light-microscope it is possible to perform two extreme estimations of the percentages of 2c, 4c and 8c hepatocytes in the suspension (see Appendix). For determination of cell size the FACS analysis was performed in the presence of 20-μm polystyrol calibration beads (Paesel, Frankfurt, FRG) and was confirmed using a Nikon Diaphot-TMD microscope with an eyepiece micrometer.

Centrifugal elutriation

20 × 10^6 vital hepatocytes were loaded in the Beckman rotor J-6B at a flow rate of 10 ml/min, at 1020 r.p.m. and 10°C. The medium contained 0.5% gelatine and 100 μg/ml DNase I to protect the cells, improve the flow conditions and prevent cell aggregation during elutriation. Six cell fractions were obtained by increasing the flow stepwise by 5 ml/min (fraction I = 15 ml/min; fraction

Abbreviations: AFB_1, aflatoxin B_1; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; γ-GTase, γ-glutamyltranspeptidase

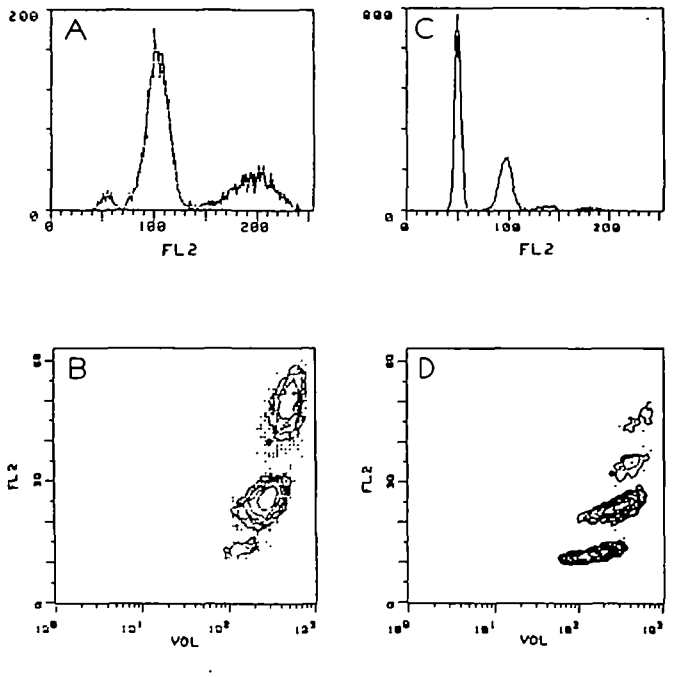

Fig. 1. Characterization of hepatocytes from untreated (**A,B**) and 2-AAF-treated rates (**C,D**) by flow cytometry. The treated animals were fed a diet containing 0.02% 2-AAF for 9 weeks. Isolated hepatocytes were stained with propidium iodide and the DNA content and volume analysed as described in Materials and methods. (**A,C**) DNA histograms; FL2 indicates propidium iodide fluorescence (arbitrary units), cell numbers are plotted on the ordinate. (**B,D**) Contour plots showing the DNA content and the cell volume; FL2 indicates propidium iodide fluorescence (arbitrary units), the volume is plotted on the abscissa (arbitrary units). The lowest contour line represents 4 cells, this number increases by a factor of 2 from each contour line to the next.

VI = 40 ml/min) and finally a seventh fraction by setting the flow to 50 ml/min. The volume of the various elutriated fractions was 200 ml; the viability of the cells in the fractions was >95% as determined by trypan blue exclusion. The cells were concentrated by centrifugation in a Sorvall RC-2B for 3 min at 750 r.p.m. using the GSA-rotor.

Results

Flow-cytometric analysis of hepatocytes from 2-AAF-treated rats

Feeding a diet containing 0.02% 2-AAF resulted in a marked increase in the percentage of diploid hepatocytes in the liver of male Wistar rats (Figures 1 and 2, Table I). The DNA histograms of hepatocytes obtained from untreated and treated animals reveal striking quantitative differences in the channels in which diploid and tetraploid cells peak (Figure 1a and c). The corresponding contour plots, which also show the cell volume, indicate that cell size and ploidy are correlated (Figure 1b and d). The increase in the number of diploid hepatocytes started after ~30 days of 2-AAF feeding. After 63 days 65–70% of the hepatocytes were diploid (Figure 2). Concomitantly the percentage of tetraploid cells (i.e. 4n mononucleated or 2 × 2n binucleated) or octaploid cells (8n mononucleated or 2 × 4n binucleated) decreased markedly. The cellular ploidy distribution of age-matched controls is shown in Table I for comparison; hepatocytes in 15- to 16-week-old animals are predominantly tetraploid. Moreover, at the age of 6 weeks, when the 2-AAF treatment was started, the cellular ploidy is very similar to that observed in the 15- to 16-week-old controls.

Not all of the diploid hepatocytes persisted after cessation of the treatment. As shown in Figure 3 a new equilibrium of the cellular ploidy classes was attained after ~130 days; at this time

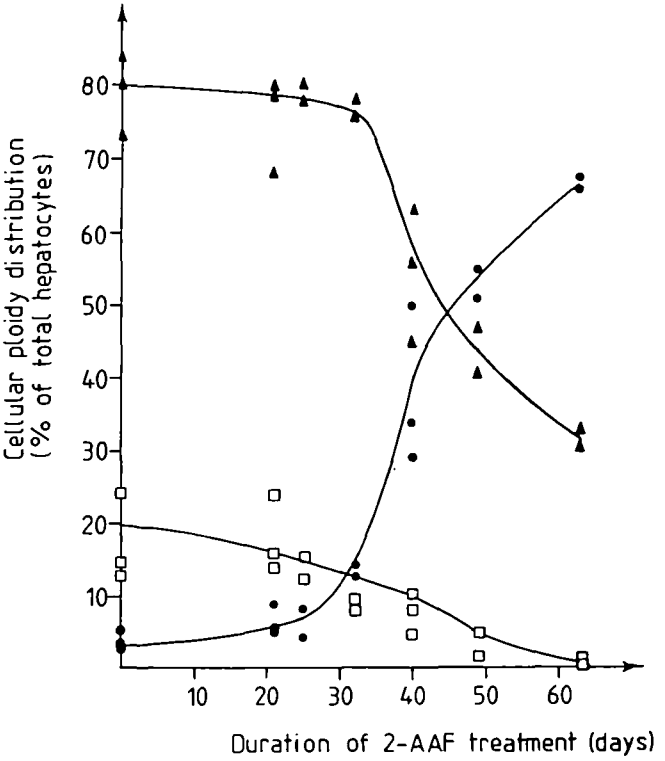

Fig. 2. Time dependence of the emergence of small diploid hepatocytes. Rats were fed a diet containing 0.02% 2-AAF for various times up to 9 weeks. Flow cytometry was performed as described in Materials and methods. (●) Diploid, (▲) tetraploid and (□) octaploid cells. Each symbol indicates the analysis of one animal.

Table I. Ploidy distribution of hepatocytes isolated from untreated and 2-AAF-treated rats

	% of hepatocytes		
	2c	4c	8c
Untreated rats (6 weeks old)	4 ± 2	81 ± 3	15 ± 4
Untreated rats (15 weeks old)	3 ± 2	79 ± 2	18 ± 2
Rats fed a diet with 0.02% 2-AAF for 9 weeks	67 ± 2	32 ± 1	1 ± 1

Values represent means (±SD) of five animals (untreated rats) and three animals (treated rat) respectively.

diploid and tetraploid hepatocytes comprised 42 and 52% of the cells respectively.

Centrifugal elutriation of 2-AAF-treated rats

Hepatocytes isolated from 2-AAF-treated animals were separated by centrifugal elutriation into seven cell fractions (Figure 4). In the first four fractions the mean volume/diameter of the hepatocytes was estimated using polystyrol calibration beads. As shown in Table II cell size increased markedly from fraction I to IV. No cell size analysis was performed in fractions V–VII, since these fractions are increasingly contaminated with cell aggregates.

Since cell size and ploidy are correlated, centrifugal elutriation also separates hepatocytes according to their ploidy. Thus, the first three elutriated fractions contain predominantly diploid hepatocytes, whereas the latter four elutriated fractions contained increasing amounts of tetraploid hepatocytes. The values of fractions V–VII could not be corrected for cell aggregates. Fixation of hepatocytes prior to the FACS analysis decreases the

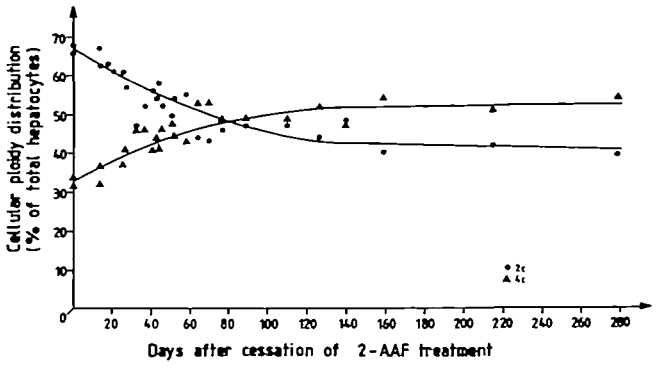

Fig. 3. Persistence of small diploid hepatocytes induced by treating rats for 9 weeks with 2-AAF. Flow cytometry was performed as described in Materials and methods. (●) Diploid and (▲) tetraploid cells. The abscissa indicates the time after cessation of the treatment. Each symbol indicates the analysis of one animal.

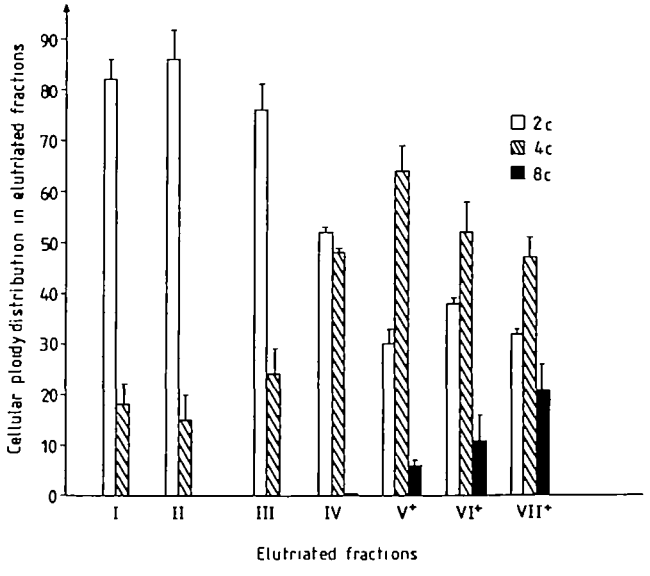

Fig. 4. Centrifugal elutriation of hepatocytes isolated from rats fed a diet containing 0.02% 2-AAF for 9 weeks. Centrifugal elutriation and analysis of the cellular ploidy distribution of the hepatocytes in the various fractions was performed as described in Materials and methods. The data represent the mean of two separate experiments. The asterisks indicate that fractions V–VII are not corrected for doublets.

number of doublets causing the values for diploid and tetraploid hepatocytes to represent over- and underestimate respectively. To determine the percentage of γ-GTase-positive hepatocytes in the various elutriated fractions, cell smears were stained according to Lojda *et al.* (11). As shown in Table II the percentage of γ-GTase-positive hepatocytes did not significantly differ between the various elutriated fractions.

Discussion

The carcinogen 2-AAF induced a dramatic decrease in the ratio of tetraploid/diploid liver cells, when rats were fed the aromatic amine for >30 days. Light and electron microscopical observation showed the new diploid cell population to have clear characteristics of hepatocytes such as a yellow-brown colour, an abundant endoplasmic reticulum and many large mitochondria (data not shown). The possibility that these cells represented non-parenchymal liver cells was also clearly ruled out since the latter are considerably smaller than the carcinogen-induced diploid

Table II. Separation of hepatocytes from 2-AAF-treated rats by centrifugal elutriation: cell size and amount of γ-glutamyltranspeptidase-positive hepatocytes in the elutriated fractions

Fraction	Mean size (μm)	% γ-GTase-positive cells
parent cell suspension	–	8.6 ± 2
I	17.2	8.6 ± 0.9
II	18.9	7.1 ± 2.6
III	20.9	7.8 ± 2
IV	22.8	8.4 ± 3.5
V	–	8.3 ± 2
VI	–	8.9 ± 1.2
VII	–	9.0 ± 1.5

Values represent means ± SD of three animals.

hepatocytes. The smallest diploid hepatocytes which accumulated in fraction I, had a mean diameter of 17–18 μm, whereas oval cells, Kupffer cells and endothelial cells have mean diameters of 13, 11 and 8 μm respectively (12,13).

The origin of the small diploid hepatocytes remains to be elucidated. One possibility is that they result from selective outgrowth of diploid hepatocytes combined with a selective toxic effect on higher ploidy hepatocytes (3,14). A second possibility is that the oval cells proliferate in the 2-AAF-treated animals (15,16) and transform to hepatocytes, a capability demonstrated by Evarts *et al.* (16).

Only about two-thirds of the diploid cells persisted after termination of the carcinogen treatment. The loss of diploid hepatocytes may be due to apoptosis (17), or may reflect binuclearization and formation of mononucleated tetraploid cells, which is the normal sequence of events during developmental polyploidization (18). No loss of diploid nuclei was observed in rat liver after termination of treatment with AFB_1 (3) or 3′-methyl-4-dimethylaminoazobenzene (1). This difference might reflect different properties of the carcinogens used, or methodical differences. In the studies cited ploidy was determined in isolated nuclei which does not allow binuclearization to be detected.

The persisting diploid hepatocytes may be relevant for 2-AAF-induced hepatocarcinogenesis. This notion is supported by the finding of Digernes and Iversen that treatment of rats with 2-AAF and phenobarbital causes the appearance of diploid hepatocellular carcinomas and adenomas (7). Similarly, Seglen and co-workers found almost exclusively diploid adenomas and carcinomas in 2/3 hepatectomized rats treated with DEN and 2-AAF (4,6). Theoretical considerations also favour a distinct role for diploid preneoplastic liver cells in hepatocarcinogenesis. For example, diploid cells provide a higher probability of expressing recessive mutations compared to tetraploid and octaploid hepatocytes (4). Other findings argue against an indispensable role for diploid hepatocytes or diploidization in hepatocarcinogenesis. Recently Sarafoff *et al.* (19) studied the DNA content of hepatocytes in ATPase-deficient putative preneoplastic foci in rat liver, induced by a single dose of *N*-methyl-*N*-nitrosourea and subsequently phenobarbital feeding. They showed that the majority of early/small foci were diploid, but that tetraploid foci also existed in the same small size class of ATPase-deficient foci. The authors concluded that diploid hepatocytes may indeed develop more easily than tetraploids into preneoplastic clones, but that tetraploid hepatocytes are also a possible target population for the transforming action of hepatocarcinogens. A predominance of diploid preneoplastic foci has also been reported by Deleener *et al.* (20) and Danielson *et al.* (21). In accordance with the notion that

diploidy may not be essential in hepatocarcinogenesis *per se*, *N*-nitrosomorpholine has been shown to increase the proportion of polyploid hepatocytes (22) and several reports describe polyploid liver tumours after treatment of rats or mice with carcinogens (cf. 21). Thus it seems that the experimental conditions may have a great impact on the ploidy distribution of preneoplastic hepatocytes and hepatocarcinomas and that certain carcinogens and treatments may especially promote diploidization of rat liver (2–4,7).

In order to characterize further carcinogen-induced diploid liver cells, we studied the percentage of putatively preneoplastic γ-GTase-positive hepatocytes after centrifugal elutriation. While cell size and ploidy clearly increased in the elutriated fractions, there was no significant difference in the percentage of γ-GTase-positive hepatocytes in the various fractions. Thus γ-GTase-positive hepatocytes obtained by feeding 2-AAF do not represent a distinct size class of hepatocytes. This contrasts with the results of Schwarze *et al.* (23) who showed a higher percentage of γ-GTase-positive cells in the diploid compared to the tetraploid cell fraction after centrifugal elutriation. However, their carcinogen treatment differed considerably from ours, since they administered 50 mg DEN/kg after 2/3 hepatectomy before feeding 2-AAF. The discrepancy between our results and theirs indicates that the characteristics of diploid hepatocytes may differ depending on the carcinogen treatment used. This may also apply to the neoplastic potential of diploid hepatocytes induced by different carcinogens.

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Appendix

Suppose for simplicity that analysis of the contour plot of a 2-AAF-treated animal gives the following values: 2c = 4000, '4c' = 5000, '6c' = 150 and '8c' = 850, i.e. a total of 10 000 events. Furthermore it has been determined that 9.5% of the cells are not single but in doublets, i.e. the hepatocyte suspension contains a total of 500 aggregates and 9500 single cells. The 150 '6c' events can be exclusively attributed to aggregates consisting of 150 diploid and 150 tetraploid cells. They have to be added to the diploid and tetraploid events, which amount after this first correction to 4150 and 5150 respectively. The remaining 350 aggregates represent doublets formed by diploid or tetraploid cells. Two extremes are possible: if *all* aggregates represent diploid cells the hepatocyte suspension would contain 4850 (46%) diploid, 4800 (46%) tetrapoloid and 850 (8%) octaploid hepatocytes; on the other hand if the doublets consist exclusively of tetraploid cells the values would amount to 4150 (39%), 5850 (56%) and 500 (5%) respectively. Since the percentages of diploid and tetraploid hepatocytes in 2-AAF-treated animals is approximately the same, it is most likely that the remaining 350 doublets comprise equal portions of diploid and tetraploid aggregates. Thus, we expressed the data as the mean of the two extreme estimations: 2c = 42 ± 3%, 4c = 51 ± 5% and 8c = 7 ± 1% (± indicates the range of the extreme estimations).

For hepatocytes from untreated animals the correction of the FACS data has to be modified. For simplicity we assume that the contour plot gives the following data: '2c' = 480, '4c' = 6520, '6c' = 20 and '8c' = 2500; again we assume that the suspension contains 500 doublets in a total cell number of 10500. After correction for the hexaploid events representing diploid/tetraploid aggregates we have '2c' = 500, '4c' = 6540 and '8c' = 2500. For further correction of the data it is reasonable to assume that the 480 remaining doublets consist only of tetraploid cells; this is suggested by the low percentage of diploid cells and the small number of hexaploid events. Thus, the corrected data are 2c = 500 (5%), 4c = 7500 (75%) and 8c = 2020 (20%).