Aneuploid and Polyploid Cellular DNA Heterogeneity in Insect Cell Material of Diptera Species Analyzed by Flow Cytometry

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Flow cytometric 1-parameter DNA analysis and 2-parameter DNA/protein analysis have been performed with cell material of the diptera species *Chironomus thummi, Drosophila melanogaster, Calliphora vicina* and *Musca domestica* using an impulse cytophotometer with a new quartz objective, that was especially manufactured for cytofluorometric investigations. The occurrence of heterogenous cell populations with aneuploid and polyploid DNA content within the cell material of different developmental stages of diptera species have been determined, whereby in larvae polyploid cell populations and in imagos aneuploid cell populations predominate. Partially separation of 2C cells from other cell populations with higher DNA content can be done by Ficoll-Hypaque centrifugation as demonstrated with cell material from *Chironomus* larvae. For flow cytometric DNA analysis of insect cell material a simple and rapid cell preparation and staining technique is presented by using the DNA-specific fluorochrome DAPI in combination with the protein fluorochrome sulforhodamine 101. Employment of flow cytometry in diptera genetics might be a new tool for cytological and cytogenetic investigations as shown with the classical genetic objects *Chironomus* and *Drosophila*.

Introduction

Flow cytometry is a sensitive and rapid evaluation of cellular DNA [1–3] and has been employed in many studies of various biological objects like microorganisms; amoebidae; plants; amphibia, reptiles and mammals [4–8]. Flow cytometric DNA analysis of animal cells demonstrates characteristic distribution histograms of relative DNA content with peaks for 2C cells (cells in G_1/G_0 phase of the cell cycle) and 4C cells (G_2+M phase). Between both peaks the amount of cells in the S-phase is recognizable [9]. Alterations in cellular DNA content can be detected with great exactness by this technique, such as described for seeds of cultivated plants [10] and tumor cells [11, 12].

In diptera species, which are classical objects in genetic investigations such as the species *Chironomus* and *Drosophila,* polyploid cells are well known in single tissues and organs. For examination of DNA content in these animals the rapid, sensitive and accurate technique of flow cytometry presents itself as an additional cytogenetic method.

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Material and Methods

Animals

The following animals have been used: larvae of *Chironomus thummi* (midge), larvae, pupae and imagos of *Drosophila melanogaster* (fruit fly), imagos from *Calliphora vicina* (blowfly) and *Musca domestica* (common house fly). For adjustment and calibration of the flow cytometer (FCM) chicken red blood cells (CRBC) *(Gallus gallus dommesticus)* have been used.

Cell preparation and staining

Animals were mechanically minced in 1 ml of 0.01 M phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 10 µg/ml 4′,6-diamidino-2-phenylindole-2 HCl (DAPI; Fa. Serva) [13] and shaken carefully for 3–5 min. Then 9 ml of 5 µg/ml DAPI and 30 µg/ml sulforhodamine 101 (SR 101; Fa. Sigma) in 0.18 M Tris-HCl buffer containing 0.2 M NaCl [14] was added for 45 min. The stained cell suspension was filtered through nylon gauze rounds (mesh size 60 µm).

For cell separation with Ficoll-Hypaque 1 ml of minced *Chironomus thummi* larvae cells was layered on 5 ml of Ficoll-Hypaque solution (density 1.077 g/ml; Fa. Pharmacia Inc.) [15] in a 10 ml

tube and centrifugated 45 min at 1000 × *g* (2300 rpm; Labofuge III; Fa. Heraeus). The upper layer, the interface layer above Ficoll-Hypaque and the pellet were harvested, washed with PBS and centrifuged 10 min at 3000 rpm. After discarding of supernatants pellets were resuspended in DAPI/SR 101 solution.

Flow cytometric measurement

Flow cytometric measurements were done with a PAS-II impulse cytophotometer (Fa. Partec AG) by using a new quartz objective (40 × magnification/numerical aperture 0.80; Fa. Partec). Filter combinations of KG 1 and BG 38 (heat protecting filters), excitation filter UG 1, dichroic mirrors TK 420 and TK 560 and the barrier filter GG 435 were used for 1-parameter DNA measurements. For 2-parameter DNA/protein analysis an additional RG 590 barrier filter was employed. The mean flow rate through the flow chamber has been about 100 cells/sec.

Results and Discussion

Flow cytometric DNA analysis of insect cells prepared from diptera species have been done successful by using the described preparation and staining procedure with the DNA-specific fluorochrome DAPI [13] in combination with the protein fluorochrome SR 101 [14] without any step of fixation. Staining cells with DAPI yielded satisfactory DNA histograms, but addition of SR 101 reduced the CV values of DNA distribution curves (CV = coefficient of variation; the standard value for the exactness of FCM measurements), such as described in investigations done with plant protoplasts and human tumor cells [6, 16]. The CV values of single DNA measurements of presented diptera species ranged from 1.9% to 4.3% (mean value = 2.9%). Employment of other DNA-specific fluorochromes (like chromomycin A_3, ethidiumbromide, propidiumiodide or mithramycin) yielded non-interpretable DNA histograms of minced cell material.

Very advantageous for the flow cytometric analysis of diptera cells has been the use of a new quartz objective (40 × magnification; numerical aperture 0.80), specially manufactured for the type of used impulse cytophotometer (PAS-II, Fa. Partec). In comparison with commonly used glass objectives the quartz objective shows a darker background, allows precise focussing of the probe stream, and therefore optical signals can be collected and converted in electronical signals with less noise. Amplification of emitted optical signals from fluorochrome stained small particles like cells from *Chironomus* or *Drosophila* (the DNA content of 2C cells from *Chironomus* larvae is a third of simultaneous DAPI/SR 101 stained CRBC, which had been used as internal standard for adjustment and calibration of the impulse cytophotometer in all experiments done with diptera cell material; the 2C DNA content of *Drosophila* larvae is a fifth of CRBC) can be done effectively with the new quartz objective.

DNA histograms of investigated diptera cells show different DNA distribution patterns than measured cells from higher animals, which demonstrate the characteristic DNA distribution curves with two peaks for 2C and 4C cells. The occurrence of a heterogeneous DNA distribution in insect cells have been determined, which indicates several cell populations with different DNA content within whole animals. In cells prepared from larvae polyploid cell populations predominate (Fig. 1a, 1b). In cell material from imagos aneuploid cell lines are detectable (Fig. 1c, 1d). During the development of diptera species from larval stage over pupal stage to imago changes in the DNA distribution occurred, as seen in investigations done with cell material from *Drosophila melanogaster* (Fig. 2a–c).

Aneuploid and polyploid cellular DNA heterogeneity in cell material from diptera is recognizable with distinctness as shown in the measurements of *Chironomus thummi* larvae (Fig. 1a). The DNA histogram presents peaks of 2C, 4C, 8C and 16C cells with linear relationship. Peak analysis indicates at least three polyploid cell populations: G_1/G_0 cells of a diploid, a tetraploid and an octoploid stem line. The 16C peak represents the G_2+M cells of the 8C cell population. Artefacts like doublets or cell clumping are minimized by using the described preparation technique as observed by fluorescence microscopy. Additional the G_1/G_0 cells of an aneuploid cell population between the 2C and 4C peak can be seen in the *Chironomus* larvae cell material. The G_2+M phase cells of this population are detectable between the 4C and 8C peak (tetraploid and octoploid cells). The 4C peak rep-

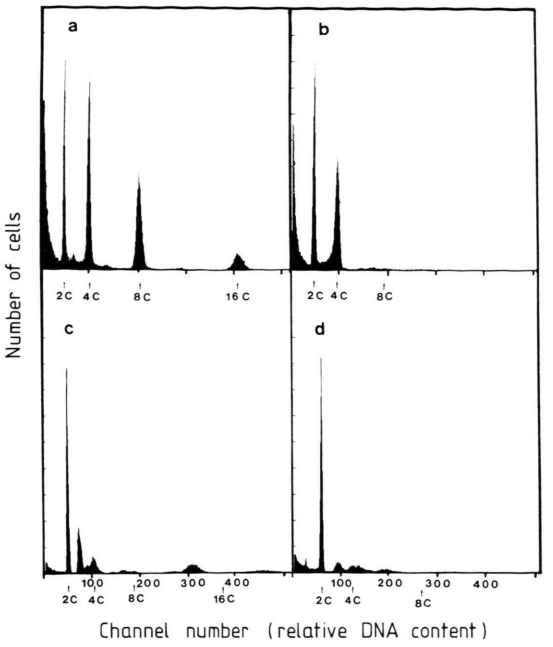

Fig. 1. DNA histograms of DAPI/SR 101 stained cells: (a) *Chironomus thummi* larvae; (b) *Drosophila melanogaster* larvae; (c) *Calliphora vicina* imago and (d) *Musca domestica* imago. Additional the C-levels in the histograms are presented.

resents 4C cells in G_1/G_0 and some 2C cells in G_2+M as well. The 8C peak contains DNA values from some 4C cells in G_2+M. This can be confirmed by partially cell separation of the 2C cell population with Ficoll-Hypaque [15] of minced cell material as shown in Fig. 3. A normal DNA distribution with two peaks is shown in cells harvested in the interface layer (III) above Ficoll-Hypaque (Fig. 3c). The layer (II) probe shows cellular debris after centrifugation (Fig. 3a) and the pellet (IV) contains all cell populations whereas the amount of the 2C cells is markly reduced (Fig. 3d).

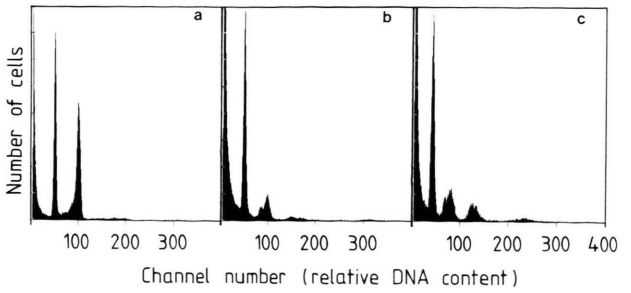

Fig. 2. DNA histograms of DAPI/SR 101 stained cells of *Drosophila melanogaster* larvae (a), pupae (b) and imagos (c).

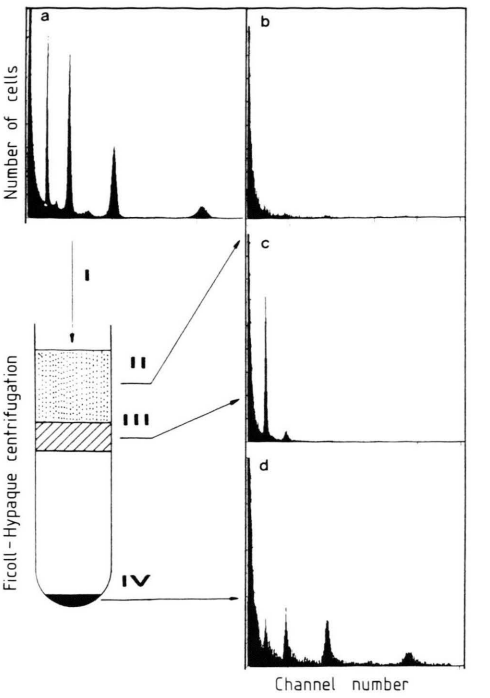

Fig. 3. Cell separation of minced *Chironomus thummi* larvae, with Ficoll-Hypaque centrifugation. DNA histograms of: (a) minced non-centrifugated probe, layered on Ficoll-Hypaque (I); (b) centrifugated layered probe, upper layer (II); (c) interface layer above Ficoll-Hypaque (III); (d) pellet (IV).

DNA analysis of *Drosophila melanogaster* larvae cells demonstrated a diploid and a tetraploid cell population (Fig. 1b, 2a). An aneuploid cell population as shown in *Chironomus* larvae cells can be detected approximately near the 4C peak. Overlapping of these cells and the 4C population occurred in this histograms, but the existence of these aneuploid cells within *Drosophila* is evident in FCM-DNA analysis of cell material from pupae and imagos as well (Fig. 2b, 2c).

Investigations with imagos from *Calliphora vicina* (Fig. 1c) and *Musca domestica* (Fig. 1d) presented a 2C cell population and several aneuploid

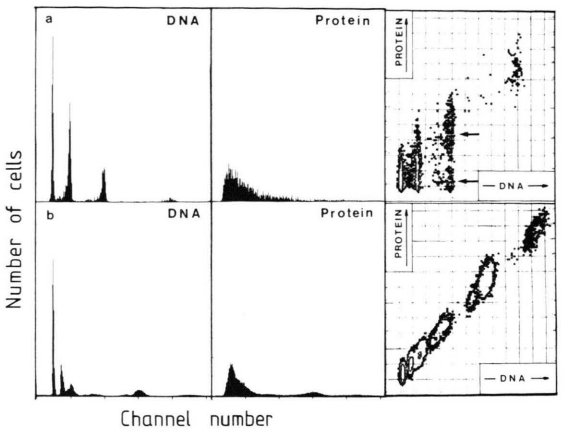

Fig. 4. 2-Parameter analysis of DAPI/SR 101 stained cells of *Chironomus thummi* larvae (a) and *Calliphora vicina* imago (b). Presented are single histograms of DNA and protein, and the bivariate contour histograms of simultaneous measured DNA and protein content. The two arrows indicate two cell populations at the 8C level in histogram (a).

stem lines. The DNA histogram of *Musca domestica* shows an additional peak with half the DNA amount of the 2C cells. These cells might be haploid cells of the tested animal (1C or C).

The results of the 2-parameter measurements of DNA and protein as shown in Fig. 4a and 4b allows an even better identification of the various cell lines [16]. Cells with the same DNA content but different protein content can be distinguished in the histogram of *Chironomus thummi* larvae cells (Fig. 4b), but not in similar measurements of *Calliphora* (Fig. 4a). Within the 8C peak two cell populations can be detected (see arrows).

The description of flow cytometric analysis of cells from different diptera species demonstrates the availability of this technique for cytological and cytogenetic investigations with insect cell material. Similar to experiments with other biological objects [4–8] flow cytometry can be used for genetic analysis of these animals. A simple and rapid cell preparation and staining method is presented, that allows measurement of cellular DNA or DNA and protein content. This analytical technique [1–3] might be a new tool for additional investigations in cytogenetics of the classical genetic objects *Chironomus* and *Drosophila*. For example influences of environmental factors on the DNA distribution in whole animals or during their development can be observed very quickly and with high precision.

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