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

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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 domesticus*) 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) 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 meshes (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.) in a 10 ml

**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 G1/G0 phase of the cell cycle) and 4C cells (G2+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 cytogentic method.

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tube and centrifuged 45 min at 1000 \times 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 \times \text{ magnification}; \text{ numerical aperture 0.80}; Fa. Partec). Filter combinations of KG 1 and BG 38 (heat protecting filters), excitation filter UG1, 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 A3, 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 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: G1/G0 cells of a diploid, a tetraploid and an octoploid stem line. The 16C peak represents the G2+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 G1/G0 cells of an aneuploid cell population between the 2C and 4C peak can be seen in the *Chironomus* larvae cell material. The G2+M phase cells of this population are detectable between the 4C and 8C peak (tetraploid and octoploid cells). The 4C peak rep-
resists 4C cells in G1/G0 and some 2C cells in G2+M as well. The 8C peak contains DNA values from some 4C cells in G2+M. This can be confirmed by partial 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. 3 c). The layer (II) probe shows cellular debris after centrifugation (Fig. 3 a) and the pellet (IV) contains all cell populations whereas the amount of the 2C cells is markedly reduced (Fig. 3 d).

DNA analysis of Drosophila melanogaster larvae cells demonstrated a diploid and a tetraploid cell population (Fig. 1 b, 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. 2 b, 2 c).

Investigations with imagos from Calliphora vicina (Fig. 1 c) and Musca domestica (Fig. 1 d) 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).

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.