

CYTO CHECK

Cytostatic Drug Testing Kit for Flow-Cytometry

partII: cytostatic drug assay



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2. Cytostatic drug assay

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2.1 Cell Preparation

2.1.1 Pleura and Ascites

Ascites or pleural fluids are cultured as obtained. Once taken from the patient, the sterile sample is kept between 0 to 4° C until the begin of incubation with the cytostatic drugs.

In case of strongly hemorrhagic samples, 50 ml cell suspension are centrifuged for 10 min at 300 x g in a sterile 50 ml plastic tube (Falcon, Becton Dickinson) with conical bottom and screw cap. The supernatant fluid is preserved in a sterile tube for later resuspension. The cell sediment is resuspended in 25 ml of sterile isotonic Percoll (Pharmacia) solution with a density of 1.089 g/ml. The isotonic Percoll solution is prepared in the following way: 10.8 ml of a 5 M NaCl solution, 1.2 ml 3 M TRIS/HCL solution pH 7.4 and 88 ml of distilled water are mixed and sterilised for 30 min at 120° C. 50 ml sterile Percoll from a freshly opened bottle of Percoll (1.132 g/ml) are added. The pH of this isotonic Percoll solution is adjusted to pH 7.35 by addition of 37 % HCL at 0° C. The density is checked by taking a sample of 1 ml with a preweighed tip of a plastic tip pipette (Eppendorf, Hamburg, FRG). The filled tip is weighed again at room temperature and the weight of the plastic tip alone is subtracted to obtain the weight of the fluid content which represents the density of the Percoll solution. Further sterile distilled water is added when the

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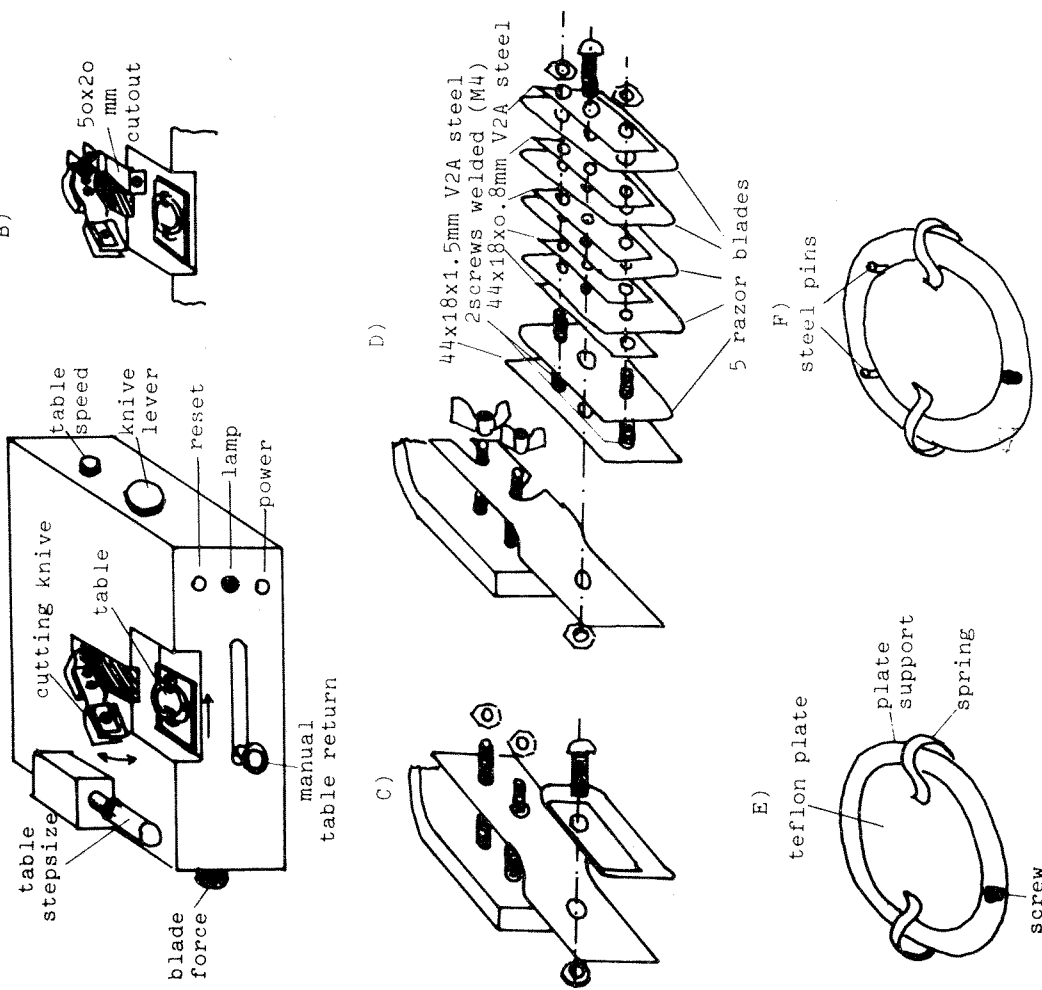


Fig. 1 Modifications of the McIlwain tissue chopper. The instrument box (A) is cut at the right side of the knife to have room for larger screws which are easier to open (B). A piece of sheet metal is fixed with two small screws to close the cutout at a 5 mm lower level than the original surface of the box. The blade assembly of the knife (C) is changed such that 5 parallel razor blades are available as cutting capacity (D). The fixation holes of the knife are opened, in addition, to permit the fast removal of the knife from the knife support without complete unscrewing. The teflon plate on the plate support (E) is better fixed against sliding by two additional steel pins (F).

density is too high. The cell suspension is centrifuged for 15 min at 200 x g which sediments the majority of erythrocytes and flotates the nucleated cells to the top of the centrifuge tube. The nucleated cells are removed from the upper 1 cm of tube, transferred into a new tube, diluted with 50 ml sterile saline buffer (TBS) (0.15 M NaCl, 10 mM TRIS/HCL pH 7.35), centrifuged for 10 min at 200 x g and resuspended in the protein containing supernatant fluid of the first centrifugation.

2.1.2 Solid tumors

Between 0.05 to 1.0 g primary or metastatic tumor tissue is removed immediately after surgery and immersed in TBS, cooled to 0° C in an ice bath and kept between 0 and 4° C during the following procedure until incubation with the cytostatic drugs. The tissue sample is put on the teflon plate of McIlwain electric tissue chopper (The Mickle Comp., Gomshall, England) and 20 to 100 μ l of sterile TBS are layered onto the tissue to avoid drying. The tissue is chopped one time. The chopper is modified such that five parallel razor blades with 0.8 mm distance are used as cutting knife instead of the one blade provided by the manufacturer (Fig. 1). The screw holes of the knife are opened to permit an easier change of the knife. We also found it useful to introduce 3 steel pins vertically into the teflon plate support such that they stand approximately 1 mm out of the support, to avoid sliding away of the teflon plate during chopping. The cutting knife and the teflon plate are sterilized for 30 min at 120° C prior to use. The chopped tissue is introduced in a 50 ml Falcon tube with a sterile spatula and resuspended in 10 ml cold TBS. The suspension contains small tissue pieces which are 30 to 50 times sucked quickly back and forth in a 1 ml Eppendorf pipette (Eppendorf, Hamburg, FRG) without producing air bubbles. The disposable plastic tip of the pipette is cut for this purpose at the bottom to obtain an opening of 0.5 or 1 mm in diameter in order to let the tissue pieces just freely pass. The cell suspension is filtered through a V2A-steel sieve with 60 μ m mesh width, washed twice by centrifugation in 50 ml

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TBS at 2000 x g and resuspended in 30 ml of 1/2 or 1/3 diluted patient serum. The serum is diluted with L-15 medium containing 10 mM HEPES or with TBS. A 3 ml sample of the cells is twice washed by centrifugation with 10 ml TBS, resuspended in 1 ml TBS, stained and measured in the flow-cytometer as indicated below to have a reference of the starting material before cell culture. No antibiotics are added to the cell suspension in case of ascites or pleural fluid and also when the tissue is taken from a sterile tumor. 250 U/ml of nystatin (Mycostatin®) were added to cell suspensions of non sterile tumors.

2.2 Cytostatic assay

12 wells of a 24 well microtiter plate are filled in a laminar flow clean-bench with 3 ml cell suspension per well. 50 μ l of cytostatic drugs are added into 10 wells, 50 μ l TBS are added to the remaining 2 wells which serve as controls. The cytostatic drugs are diluted with sterile saline such that the final drug concentration is between 5 to 10 times the peak plasma level achievable in vivo. The following final assay concentrations are typically used: Doxorubicin (Adriblastin®) 2 μ g/ml, 4-hydroxyperoxy-cyclophosphamid (active metabolite of Endoxan®) 75 μ g/ml, Cisplatinum 2 μ g/ml, Methotrexate 2 μ g/ml, 5-Fluoruracil 30 μ g/ml, Vindesinsulfate (Eldesine®) 160 ng/ml, Vinblastinsulfate (Velbe®) 300 ng/ml, Actinomycin-D (Dactinomycin®) 100 ng/ml, Thiotepa 100 μ g/ml. Another approach, mainly suited for automated flow-cytometers, is to reduce the assay volume to 1 ml cells and to test each cytostatic drug at 0.1, 1 and 10 times the peak plasma concentration. The microtiter plates are cultured for 4 to 7 days at 37° C under saturating humidity conditions in air with 5% CO₂. It is also possible to culture under reduced oxygen tension in an incubator with constantly controlled O₂ and CO₂ partial pressure (Labotect, Göttingen, Germany). The cells are mechanically loosened from the bottom of the wells after the end of the culture period by scraping and sucking back and forth the content of each well with the plastic tip of an 1 ml-Eppendorf pipette, transferred to

a 10 ml plastic tube with conical bottom, filled up to 10 ml with TBS, centrifuged at 200 x g for 10 min, washed another time with 10 ml TBS and resuspended in 1 ml TBS.

2.3 Cell staining

250 μ l cell suspension are transferred into an Eppendorf plastic cup (1.5 ml). 5 μ l of staining cocktail, containing 1,4-diacetoxy-2,3-dicyano-benzene (ADB, MW 244), 1 mg/ml, propidium iodide (PI, MW 668) 2 mg/ml in dimethyl-formamide (DMF) and DCH stained, monodisperse calibration particles (size 60 μ m³, diameter 4.88 μ m) at a concentration of 1 x 10⁷ particles/ml are added.

If two parameter histograms are measured, two 250 μ l aliquots are taken from each assay. The first is stained with 5 μ l ADB alone and the second with 5 μ l PI alone. The staining time for all assays is 5 min at room temperature.

2.4 Flow-cytometric measurement

The flow cytometric measurement is performed as indicated above.

2.5 Data evaluation

The data evaluation is similar as described above with the exception that 4 areas are delimited in the cell volume versus esterase activity histogram (Fig. 2) of the vital cells. The areas indicate the calibration particles, the inflammatory cells, the tumor cells with high and low esterase activity. The absolute concentration of the cells in compartments b to c of Fig. 2 can be calculated since the concentration of the particles in the assay is known (2 x 10⁵/ml).

The further evaluation of the three parameter cube is similar to the evaluation of the ADB/PI cell staining assay (see above). The display of the intracellular pH

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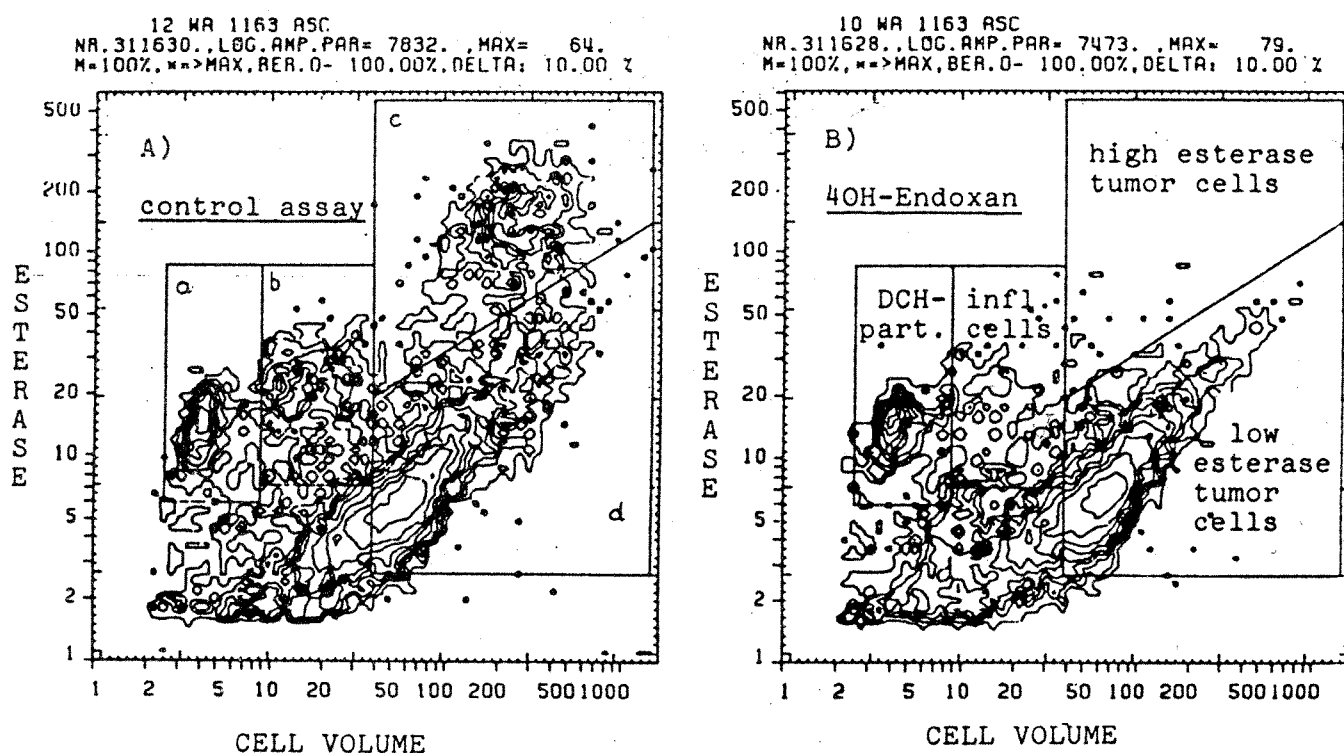


Fig. 2 Control assay (A) and 4-Hydroxyperoxy-cyclophosphamid (4 OH-Endoxan) (B) assay. The tumor cells with high esterase activity decrease significantly in th 4 OH-Endoxan assay.

distribution, the esterase activity distribution of the vital cells and of the cell volume versus DNA histogram of the dead cells are used to extract a maximum of information on the vital and dead cells of each measurement. The drug sensitivity diagram (**onkobiogram**) (Fig. 3) of each patient is calculated from the 12 cultured assays and the assay of the initial cell suspension, in the following way: The absolute concentrations of inflammatory and high activity tumor cells of the control assays are set as 100% and the other assays are related to the control assays. Furthermore the ratio of inflammatory over high activity tumor cells of each assay is calculated as **therapeutic index**. A high therapeutic index indicates preferential killing of tumor cells and conservation of the inflammatory cells. The low activity tumor cells are mostly dead cells. They consist of a shrunken plasma membrane without signs of a nucleus. The cell ghosts pick up only between 5 to 10% of the DCH stain of a vital cell (Fig. 2 a, b). They do also not appreciably stain with the DNA/RNA dye acridin orange and the esterase dye fluorescein-diacetate.

The minimum requirement for the calculation of the onkobiogram is a two parameter cell volume or forward light scatter versus esterase activity histogram. Such histograms can be measured and displayed in all flow-cytometers or sorters currently available.

It is important to note that the described assay (1) tests the resistance of cells against cytostatic drugs but does not primarily diagnose malignancies. The assay can only be used as a diagnostic tool to recognize aneuploid cancer cells in the cluster of large cells in the cell volume/DNA histogram (Fig. 5 c). The small cells of the assay are inflammatory cells i. e. lymphocytes and granulocytes. The granulocytes die away during one week of culture, while the lymphocytes remain and sometimes increase in number. The large cells are mostly tumor cells as can be seen from the cell volume/DNA histogram of aneuploid tumors. 59% of colo-rectal cancers are aneuploid according to our methodology (2).

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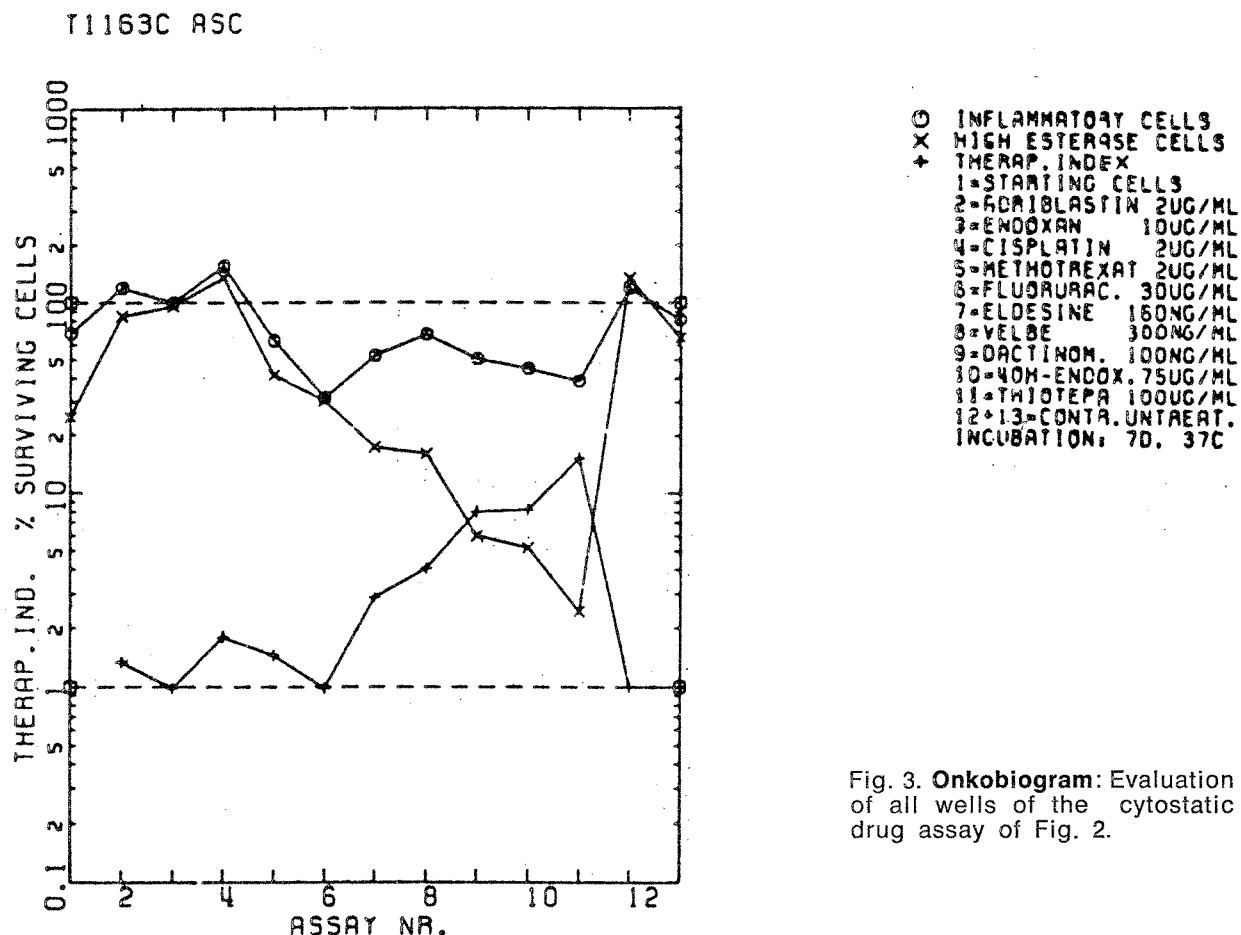


Fig. 3. **Onkobiogram**: Evaluation of all wells of the cytostatic drug assay of Fig. 2.

2/3 of the large cells of such tumors were in these cases aneuploid tumor cells. The reduction of the large cells by more than 50 % in the cytostatic drug assay of solid tumors (3) is, therefore, an indicator of tumor cell sensitivity against a cytostatic drug. Mesothelial cells in ascites and pleural effusions cannot be clearly distinguished from tumor cells by the volume measurement. This, may be later possible by using an additional parameter as FITC-labelled antibodies against tumor associated cell surface antigens. The ovarian and cervix cancers which are often the primary source for the tumor cells in malignant ascites and pleural effusion are in addition less frequently aneuploid. The diagnosis of the malignancy of an effusion has, therefore, to be established morphologically by differentiating stained smears or cytocentrifuge slides of the cells.

Literature References

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