11th Heidelberg Cytometry Symposium

Annual Meeting of the
German Society of Cytometry e.V.

Heidelberg, 22nd - 24th October 1998

Location

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German Cancer Research Centre Heidelberg
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Organisation

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Program Committee

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A. Tárnok, M. Trendelenburg

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LECTURE

ABSTRACTS

CHROMOSOMAL CHANGES DETECTED BY COMPARATIVE GENOMIC HYBRIDIZATION IN ADVANCED STAGE MALIGNANT MELANOMAS

ZSUZSA ÁDÁM[1], MARGIT BALÁZS[1], ÁGNES BÉGÁNY[2], JÁNOS HUNYADI[2] AND RÓZA ÁDÁNY[1]

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taneous malignant melanoma is a potential fatal form of skin cancer, whose cidence is rising in many regions of the world. It is assumed, like other solid mors, melanoma progress towards metastatic disease through accumulation of ultiple genetic aberrations. The aim of our study was to identify genomic anges underlying the rapid progression and the aggressive behaviour of the ease. Therefore we used comparative genomic hybridization (CGH) to screen r DNA copy number alterations in 15 primary and 8 metastatic tumors. Our sults are consistent with previous chromosome aberrations detected by romosome banding studies, demonstarting frequent alterations of chromosomes 6, 7, 9 and 10. However other genetic changes (e.g. repeated gain of hromosome 8q, recurrant high level amplification of chromosome 1p, deletion at p) were also identified. Some of these regions were previously not detected by onventional cytogenetic analysis. The underrepresentation of chromosome 9p21 nd 10q were characteristic for both primary and metastatic tumors. The most equent overrepresented region region was 6p, 60% of primary and 63% of etastic tumors showed this alteration by CGH. Four primary and two metastatic mors showed nonrandom gains on chromosme 1q, 6p, 7p and 8q. Distinct high vel amplified regions were found on 1p13, 1p21-pter, 4q11-q12, 7p12-p13, 0q13-qter and Xq21-qter, these loci may cover oncogenes which are important in elanoma progression and metastatsis formation. The progression of primary umor to metastasis was also studied in two patients. Beside common genetic hanges, which are indicative for the clonal origin of the primary and metastatic umors, new alterartions were also detected in both cases. Further analysis of ommon area of DNA losses and gains could help to identify genetic markers, hich would help to predict the aggressiveness of this neoplasm.

Comparison of Apoptosis as Detected by Annexin-V and Fluorescein - Diacetate

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Apoptosis was induced in HL-60 cells by radiation, topote-can, ara-C overcowded culture. Cells were stained with propidium-iodide (PI)/ Annexi (ANV) according to the manufacturer's prescriptions. In parallel, PI was combi with nanomolar centrations of fluorescein- diacetate (FDA), resulting in sta luorescence without the necessity to remove excess reagent.Forward sideward scatter and the two fluorescence signals were acquired by f cytometry. In 4-decade log-plots both staining procedures were able to distingu three populations: vital (PI-/ ANV+; PI-/FDA+),apoptotic PI-/ANV+;PI-/FDA-) a dead (PI+/ANV+; PI+/FDA-). The proportions of these three populations w essentially the same with both staining procedures. Also with respect to th morphology as detected by lightscatter,the three populations were alike. T suggests that (1) an arrest in FDA metabolism is accompanied by th alterations that are detected by ANV, i.e. externalization of phosphatidylseri the cell membrane, or (2) ANV detected membrane alterations are accompan by an arrest in membrane transport of FDA.

Charakterisierung der Zusammensetzung von Phyto-Planktonpopulationen mit Hilfe der Durchflußzytometrie

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Charakterisierung von Phytoplanktonpopulationen im Freiland (Süßwasser) wird Rahmen der Gewässerüberwachung routinemäßig meistens der Chlorophyll a-halt bestimmt und durch mikroskopische Untersuchungen ergänzt. Da letztere sehr aufwendig sind, erhebliche Fehlerquellen bergen und qualifiziertes Personal ordem, gibt es das Bestreben, einfachere Methoden zur Erfassung der sammensetzung zu entwickeln. Außer den lipophilen Carotinoiden und dem lorophyll kommen in einigen Phytoplankton-Gruppen hydrophile Phycobiliprotein-asse vor. Phycobiline bieten sich wegen ihrer fluoreszierenden Eigenschaften als liche Marker für die Durchflußzytometrie an.

15 Phytoplankton-Reinkulturen aus 5 Gruppen wurden Fluoreszenz-issionsspektren bei 514 nm Anregung aufgenommen. Es konnten 4 rakteristische Fluoreszenzmaxima beobachtet werden: ~585 nm (Phycoerythrin anobakterien), ~620 nm (Phycoerythrin Cryptomonas), ~650 nm (Phycocyanin anobakterien) und ~680 nm (Chlorophyll a). Im Anschluß wurden die gleichen ezies - ebenfalls bei 514 nm Anregung - im Durchflußzytometer untersucht, wobei e 4 Fluoreszenzen und die Vorwärtsstreuung gemessen wurden.

wartungsgemäß konnten Arten mit unterschiedlichen Emissionsspektren sehr gut terschieden werden, wie auch diverse Mischungen der Reinkulturen deutlich achten. Da zusätzlich zu den Spektren die Signalgröße pro Partikel und ein reuungsparameter zur Verfügung standen, ergab auch ein guter Teil der Spezies mit nlichen Fluoreszenzen distinkte Punktehaufen. Bei unbehandelten Freilandproben s der Bleilochtalsperre (Thüringen) konnten einzelne Spezies (Cryptomonas, crocystis) anhand der Zytometerdaten mit den Referenzen aus dem Labor zur ckung gebracht werden. Durch Sortieren und anschließendes Mikroskopieren rde diese Zuordnung bestätigt.

e die verwendete Anregungswellenlänge nicht direkt das Chlorophyll a anregt, nnten die gefundenen Chlorophyll a-Fluoreszenzen auch nicht als Maß für den hlorophyll a-Gehalt dienen. Die Methode könnte durch eine zusätzliche direkte nregung des Chlorophyll a (2.Laser) optimiert werden. Dabei würde gleichzeitig eine anze Reihe Phycobilin-freier Spezies mit unterschiedlichen Chlorophyll a-nregungsspektren unterscheidbar.

THE COMET ASSAY TO STUDY COLD DNA REPAIR IN B-LYMPHOBLASTS AND IDENTIFICATION OF PERITONEAL MACROPHAGES AFTER UV-A IRRADIATION

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UVA-light (320 nm-400 nm) is known to cause indirectly DNA damage in biologi cells, such as alkali-labile sites and single strand breaks.
The comet assay is a sensitive and rapid method for detection of DNA sin strand breaks in individual cells, based on migration of DNA molecules in electric field. When DNA single strand breaks are induced, e.g. by UV-A radiati the short molecules migrate faster than the unfragmented DNA. After fluorescen staining the nuclei has the appearence of a comet, with a head and a tail, there the fragmentated DNA molcules move into the tail region.

The comet assay has been applied to investigate UV-A radiation (365 n induced DNA damage a human B-lymphoblast cell line as well as the temperatu dependence from 4° C to 44° C of DNA repair. Unexpextedly similarly efficie DNA repair kinetics was found at all temperatures below 37° C, particularly in t cold at 4° C.
Furthermore the comet assay has been adapted to identify peritone macrophages within a heterogenous mixture of peritoneal exudate cells. For t purpose, the comet assay was combined with fluorescence microscopic inspecti of nuclei shape. The macrophages could be selected by their typical size a shape of their nuclei during fluorescence microscopy. The tidious separation macrophages before studies on DNA damage is no longer necessary. As application, UV-A sensitivity of reseident and stimulated macrophages w studied. The resident macrophages were more sensitive to UV-A radiation th the stimulated ones. But DNA repair of these lesions is processed by the sa time course for both cell types.

FULLY AUTOMATED FLUORESCENCE IMAGE CYTOMETRY

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luorescence microscopy is currently one of the most important tools for the amination of single cells and cellular constituents. To obtain reliable data, high mbers of cells have to be analyzed due to the large variability of the biological aterial as well as the evaluation errors of the investigator. As a consequence the evelopment of automated cell analysis is useful and has been considerably dvanced recently.
usually, stage movement for x,y and z-positioning is required to provide automatic oning of larger scan areas than one microscope field. Sometimes even more an one slide has to be analyzed in order to gather enough data. This makes utofocusing necessary, which can be a non-trivial task, especially for luorescence microscope systems. Recently, we developed a special analogue utofocus unit, which is based on image content information. The total focusing ocedure is fast (1-2 sec) and the unit was tested successfully with different kinds biological specimens.
In this contribution, we present an automated analysis system, which is based on athematical morphology and needs minimal human interaction. To enhance valuation speed we have introduced parallel processing of data under Windows T. Image analysis mainly consists of three parallel software tasks distributed to hree different hardware components. The searching and grabbing part (running n the autofocus hardware unit), the so-called image stack (within the memory of PC host) and the recognition and analysis task (running on the host CPU).
The main parts of image pre-processing, segmentation, feature classification and uantification will be introduced and discussed.
Furthermore, we present some results of automated comet assay analysis tained by this system.

Three-Dimensional Reconstruction of Interphase Chromosome Domains Topology with Laser Scanning Microscopy and Image Analysis

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During the past decade 3-dimensional image processing has become an important key component in biological research mainly due to two different developments. The first is based on an optical instrument, the so-called confocal laser scanning microscope, allowing optical sectioning of the biological specimen. The second is a biological preparatory method, the so-called FISH-technique (Fluorescence-In-Situ-Hybridization), allowing labeling of certain cellular and sub-cellular compartments with highly specific fluorescent dyes. Both methods make it possible to investigate the 3-dimensional biological framework within cells and nuclei .

Image acquisition with confocal laser scanning microscopy must deal with different limits of resolution along and across the optical axis. Although lateral resolution is about 0.7 times better than in nonconfocal arrangements, axial resolution is more than 3- 4 times poorer than that of the lateral (depending on the pinhole size). For 3D reconstruction it is desirable to improve axial resolution in order to provide nearly identical image information across the 3 dimensional specimen space. This presentation will give an overview of some of the most popular restoration and deblurring algorithms used in 3D image microscopy.

After 3D image restoration, segmentation of certain details of the cell structure is usually the next step in image processing. Different kinds of algorithms for the segmentation of chromosome territories in interphase cell nuclei will also be presented.

The segmented image regions provided the basis for chromosome domain reconstruction as well as for regional localization for subsequent quantitative measurements. As a result the chromatin density within certain chromosome domains as well as some terminal DNA sequences (telomere signals) could be measured.

Assessment of Viability of Microorganisms Emplying Fluorescent Techniques

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Viability assessment of microorganisms is relevant for a wide variety of applications in the food industry, including evaluation of inactivation treatments, and quality assessment of starter cultures for beer, wine, and yoghurt production. Viable cells can be defined as those cells which are capable of performing all cell functions necessary for survival under given conditions. The elementary requirements for viable microorganisms to survive are: **(i)** an intact cytoplasmic (plasma) membrane which functions as a barrier between the cytoplasm and the extracellular environment, **(ii)** DNA transcription, and RNA translation, **(iii)** generation of energy for maintenance of cell metabolism, biosynthesis of proteins, nucleic acids, polysaccharides, and other cell components, and, eventually, **(iv)** growth and multiplication.

Usually, the ability of cells to reproduce is considered as the benchmark method for determination of viability, and this is most commonly determined by the plate count method. The time needed to form visible colonies, however, is relatively long. Moreover, microorganisms which do not form colonies, because they are dead, sublethally damaged, viable but non-culturable, dormant, inactive, etc., are not counted. Therefore, there is an increasing interest in the development of rapid methods for the determination of cell viability. Parameters other than reproduction which can be exploited to determine the viability of microorganisms are membrane integrity, changes in cell morphology, presence of enzyme activities, respiration, membrane potential, and the intracellular pH (pH_{in}). In this study, the pH_{in} of yeasts and lactic acid bacteria were determined using pH dependent probes such as carboxyfluorescein (cF) and 5-(and-6)-carboxyfluorescein succinimidyl ester (cFSE). These probes can be taken up by the cells in the form of non-fluorescent diacetate esters, which are subsequently hydrolyzed by esterases to the fluorescent form in the cytoplasm. In this way the effect of environmental stress conditions and antimicrobial compounds on the pH_{in} of microorganisms could rapidly be determined. In particular, our work is focused on measuring the pH_{in} of *Saccharomyces cerevisiae* using fluorescence ratio imaging microscopy (FRIM). This technique allows analysis of individual yeast cells with high spatial and temporal resolution.

INTERAKTION VON REZEPTOR-TYROSIN-KINASEN (RTK) AUS DER EGFR-FAMILIE BEI UROTHELIALEN TUMORZELLEN

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Aus der Familie des Epidermalen-Wachstumsfaktor-Rezeptors (EGFR) sind wenigstens vier Mitglieder von Rezeptor-Tyrosin-Kinasen (RTK) mit unterschiedlicher Ligand-Bindungsspezifität beschrieben, die ein definiertes Expressionsmuster auf normalem, nicht transformiertem Urothel zeigen. Bei urothelialen Tumoren hingegen findet man häufig eine Überexpression eines Rezeptortyps bzw. eine abnorme und diffuse (Ko-)Expression von RTK (3), die offensichtlich das tumorbiologische Wachstumsverhalten regulieren.
Die zellphysiologische Reaktion nach Ligand-Stimulation der RTK ist von der Interaktion von Rezeptoren aus der EGFR-Familie (Ausbildung von Rezeptor-Homo- bzw Heterodimeren) und den nachfolgenden Ereignissen abhängig.
Anhand eines *in vitro* Modells verschieden differenzierter Urothelkarzinomzellinien (J82 / RT4) wurde die Koexpression verschiedener RTK aus der EGFR-Familie flußzytometrisch quantifiziert (1,2) und die spezifische Rezeptor-Internalisierung nach Ligand-Stimulation durch EGF bzw. Heregulin erfaßt. Zur Untersuchung der Rezeptor-Interaktionen wurden Fluoreszenz-Resonanz-Energie-Transfer Experimente (FRET) mit Phycoerythrin (R-PE) als Donor- und Cyanin-5 (Cy5) als Akzeptorfarbstoff durchgeführt. Eine Rezeptor-Kreuzaktivierung wurde durch proteinchemische Analysen untersucht.
Die Daten zeigen, daß die mitogene Stimulation der Tumorzellen durch einen Rezeptor-spezifischen Liganden vom Verhältnis der Rezeptor-Koexpression abhängig ist und daß der erbB2-Rezeptor eine Schlüsselrolle für die Signaltransduktion über die Zellmembran zu spielen scheint. Dieser Rezeptor spielt offensichtlich eine Mittlerrolle für die Ausbildung verschiedener Rezeptor-Aggregate innerhalb der EGFR-Familie.

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ANALYSING GENOMES BY FISH: THE IMPACT OF THE HUMAN GENOME PROJECT.

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FISH, using chromosome paints and specific DNA library clones hybridised to cytogenetic material, is now fully established as key methodology in the analysis of genomes and their rearrangements. However, recent acceleration in the progress of the Human Genome Project ??? future for genome analysis.
The plan to determine the complete human sequence map by an international collaborative effort was outlined during 1995. The agreed aim is to produce the sequence chromosome by chromosome. This ensures that useful material is produced in an essentially complete form from the very first year of the programme. It also provides the optimal way to co-ordinate international collaboration incorporating the efforts of both large and small groups, with all efforts positioned as far as possible on a publicly available map.
The Sanger Centre is currently funded by the Wellcome Trust to sequence at least 1,000 Mb of the human genome by 2005. The strategy proposed is to construct a map with fully integrated levels of increasing resolution, from the framework map to the minimally overlapping set of bacterial clones for sequencing. Each bacterial clone is subjected to shot sequence analysis followed by several rounds of finishing to yield a product that is 99.99% accurate.
In this presentation, I will review how FISH has been useful in the analysis of genomes and their rearrangements and how it is integrated into the sequencing effort of the Sanger Centre. I will highlight the uses of flow sorted chromosomes, digital microscopy and multi-colour FISH and describe how the emerging methodologies of microsphere assays, DNA array technology and matrix CGH may affect molecular cytogenetics in the future.

Propidium Iodide is a Better DNA Stain then TO-PRO-3 Iodide when Used for Four Colour Multiparameter DNA Flow Cytometry.

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TO-PRO-3 Iodide has recently been introduced as a novel DNA stain suitable for flow cytometers with an Helium-Neon or red-diode laser excitation facility. We compared TO-PRO-3 Iodide (TP3) and Propidium Iodide (PI) for four colour multiparameter flow cytometry of human solid tumours using a FACScalibur flow cytometer, equipped with the Argon-ion laser (488 nm) and a red-diode laser (633 nm).

Firstly, the effect of a broad range of different PI and TP3 concentrations on the coefficient of variation (CV) and stoichiometry of the DNA histograms was studied using paraformaldehyde fixed lysolecithin permeabilized peripheral blood lymphocytes (PBL), SIHA and HELA cervical cancer cells. Then, the optimal DNA stain was applied to freshly isolated cells from human cervical cancers, which were also stained for three cellular antigens (two surface and one cytoplasmic or one surface and cytoplasmic).

Overall, PI gave better CV's then TP3. The optimal concentration of PI ranged between 50 to 100 µM for all cell lineage tested. CV's were 1.76 +/- 0.02 (PBL), 3.16 +/- 0.014 (HELA) and 2.50 +/- 0.02 (SIHA). The optimal concentration of TP3 was 2.0 µM for PBL's CV's were (CV = 2.58 +/- 0.07), and was 0.25 µM (CV = 5.16 +/- 0.28) and 0.5 µM (CV = 3.96 +/- 0.18) for HELA and SIHA cells, respectively. A negligible difference was found between the stoichiometry of the DNA histograms from optimal PI and TP3 stained HELA and SIHA cells. A final concentration of 100 µM PI gave the best results and was successfully applied to fresh tumour cell suspensions stained for HLA expression (APC fluorescence), keratin expression (R-PE fluorescence), CD45 (FITC fluorescence) or vimentin (FITC fluorescence) and DNA.

We conclude that PI is a superior DNA stain compared to TP3, giving better CV's and comparable G_2M/G_1 ratio's, when applied to PF fixed and lysolecithin permeabilized human cervical cancer cells and PBL stained for DNA. Low CV's are of importance for the study of intra-tumour phenotypic heterogeneity, especially in cases with closely related multiple DNA stem lines or in cases with a DNA index close to 1.0. Four colour high resolution multiparameter DNA flow cytometry can be performed using a FACScalibur and PI as DNA stain and FITC, R-PE and APC as reporter molecules.

Synchronous Release of Gamma Radiation-Induced G_2 Phase Arrest by Caffeine Without Apparent Apoptosis or Necrosis

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Treatment of asynchronous HeLa cells and normal, human fibroblast (HSF) cells, with caffeine immediately following gamma irradiation attenuates the arrest at the G_2 phase checkpoint. Previously we showed that similar treatment of HeLa cells with the kinase inhibitor, staurosporine, also reduces radiation-induced G_2 arrest; however, staurosporine treatment was notably ineffective in normal HSF cells (Exp. Cell Res. 233:118-127, 1997). These results indicated that G_2 arrest might be regulated by different mechanisms in normal and transformed cells. In the present studies, we have used flow cytometric and biochemical approaches to investigate the effects of the two drugs on p53-null, HL-60 cells arrested in G_2 at 16 h after irradiation with 5 Gy. Addition of caffeine to 1.8 mM completely relieved the radiation-induced G_2 block in 3 h, while the untreated, irradiated population remained significantly blocked in G_2, and only a 25% reduction in this subpopulation was noted at 8 h. The rapidly-induced cell division in the caffeine-treated population was not accompanied by either apoptotic or necrotic cell death, and the divided G_1 cells synchronously entered S phase at 6 h after addition of caffeine. Biochemical studies showed that caffeine caused rapid hyperphosphorylation of cdc25B phosphatase, dephosphorylation of cdc2 phosphotyrosine, and activation of the cyclin A and cyclin B dependent kinases during the first 1-2 h. Thereafter, the cyclin dependent kinase activities rapidly declined, as cdc25B phosphatase, cyclin A, and cyclin B were degraded. The changes in the cellular levels of the cyclins were monitored both biochemically, in the whole cell populations, and by flow cytometry throughout the cell cycle using immunofluorescent cyclin detection coupled with BrdUrd incorporation. Treatment of irradiated cells with staurosporine was much less effective in relieving the G_2 arrest.

Supported by the US Dept. of Energy and Los Alamos National Laboratory Flow Cytometry Resource (NIH Grant p41-RR01315 and NIH Grant R01 RR06758).

Flow Cytometric Counting of CD34+ and Other Immunolabelled Cells

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Besides the determination of the percentage of cells in the different lymphocyte subclasses the precise measurement of the concentration of immunolabelled cells is of increasing interest for diagnostic purposes and in transplantation medicine.
Great effort has been spent by international groups of scientists and clinicians to overcome intra- and interlaboratory quality differences. There is still a need to establish standard procedures which are widely accepted. In case of the concentration measurement of rare cells like CD34+ lymphocytes serious technical problems exist which are not perfectly taken care of by the present commercially available counting devices.
Two procedures are offered for concentration measurements of immunolabelled cells:

1. Reference particles of known concentration are added to the blood sample. This allows a relative counting of immunolabelled cells. The advantage of this procedure is that fewer critical pipetting steps are needed.
2. The volumetric method counts all events in a precisely measured sample volume. The advantage of this procedure is that no reference particles or independent counting procedures are needed.

For this second procedure we have developed a volumetric procedure and fast real time acquisition software which has a dead time of 25 µsec per event. This short recognition time for each individual cell and the absence of any computer interrupts allows to count immunolabelled cells in a particular sample volume. In order to reduce error by pipetting sample and antibodies a sample automate performs the immunolabelling, lysing and automatic sample feed-in into the cuvette of the flow cytometer. The counting results are displayed in fractions of lymphocyte subclasses as well as in cells per ml in each corresponding quadrant of each data set.
The procedure is capable to count any type of immunolabelled cells per ml. In particular, CD34+ cell counting can be performed routinely in a fully automated procedure with high
precision.

Conformational Activity of the CD14 / ß2 - Integrin Complex by LPS and Ceramide as Analysed by Fluorescence Resonance Energy Transfer (FRET)

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Monomeric bacterial lipopolysaccharide (LPS) similar to ceramide is rapidly delivered from the plasma membrane to an intracellular site and the response of leukocytes to LPS is blocked by compounds inhibiting vesicular transport. Ceramide, furthermore, mimicks some characteristics of LPS in different in vitro models in respect to patterns of activation, suggesting that LPS and/or ceramide dependent cell activation is also transduced via the same receptor (e.g. CD14). Stimulation of the IL-6 production pathway by ceramide and LPS, however, has been shown to occur also in absence of CD14 expression. The purpose of the present study was to characterize the involvement of CD14 in cellular responses to LPS and ceramide using fluorescence resonance energy transfer (FRET) for the examination of the activation dependent assembly of CD14 with the functionally associated receptors ß2-integrin (CD11b) and the integrin associated protein (CD47).
In order to be able to analyse the association of CD14 and CD11 b or CD47, human monocytes were labeled simultaneously with -phycoerythrin (PE) conjugated antibodies directed towards CD11b or CD47 while CD14 was labeled with Cy5 as a biotin/streptavidin complex. When analysed at 488 nm excitation the emission in the spectral range of Cy5 is a measure for the proximity of both fluorochromes due to Förster-type energy transfer. In resting cells no significant energy transfer occured indicating no close association between the LPS receptor and the integrin or the integrin associated protein. LPS incubation but not stimulation with PMA or FMLP induced a significant energy transfer in the range of 20 % between both pairs of monoclonals suggesting a conformational change of the receptor complexes following ligand binding. Similar to the LPS dependent response ceramide incubation led to a significant energy transfer between CD14/11 b and CD14/47, independently of subtypes of ceramide (C2 or C24). Both stimuli, LPS and ceramide, also were highly comparable regarding maximum response that already appeared after 15 minutes and was stable during prolonged incubation.
These data prove ceramide similar to LPS as a ligand that also induces sustained conformational changes of the CD14 associated receptor complex. FRET analysis in general seems to be a sensitive method to determine receptor association and conformational changes during cell activation.

Nachweis und Charakterisierung von Einzelnen Tumorzellen im Peripheren Blut bei Patienten mit Malignen Erkrankungen.

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Der Nachweis von freien Tumorzellen ist ein wichtiger und unabhängiger Prognosefaktor bei malignen Erkrankungen, insbesondere beim Mammakarzinom. Intrazelluläres Zytokeratin 8/18 ist ein spezifischer Marker für Zellen epithelialen Ursprungs und ermöglicht so eine zuverlässige Detektion der Tumorzellen.
Zum Nachweis einzelner Tumorzellen ist die Kombination von MACS (Magnetic Activated Cell Sorting) und Durchflußzytometrie (FACSCalibur) verwendet worden, die es ermöglicht, Zellen in einer Konzentration von 1:10 Millionen aufzukonzentrieren und zu detektieren.
Im Rahmen unseres Projektes wurden 18 Patienten in den unterschiedlichsten Stadien verschiedener maligner Erkrankungen auf zytokeratinpositive Zellen untersucht. Weiterhin screenten wir 14 Normalprobanden auf falsch positive Ereignisse.
Für die Untersuchungen wurden pro Patient ca. 8 ml peripheres Blut in EDTA entnommen und innerhalb der nächsten 24 Stunden verarbeitet.
Die Anreicherung der Leukozyten fand mittels Buffy Coat statt. Im Anschluß wurde die Permeabilisierung (gleichzeitige Erythrozytenlyse) und Fixierung durchgeführt. Nach der magnetischen Markierung wurden die Zellen fluoreszenzmarkiert (CD45-PE / Anti-Zytokeratin 8/18/19 - FITC). Zusätzliche mAk zur weiteren Charakterisierung der Tumorzellen wurden im Austausch oder zusätzlich während des gleichen Färbeschrittes hinzugefügt. Die durchflußzytometrische Analyse, das Sorting und die Auswertung wurden auf einem FACSCalibur unter Cellquest durchgeführt. Im Anschluß wurden die gesorteten Zellen mittels einer konventinellen Immunozytochemie gefärbt und mikroskopisch ausgewertet.
Bei den Normalprobanden (n=14) konnten im Durchschnitt 0,9 +/- 1,0 falsch positiven Ereignisse nach einer Anreicherung detektiert werden. Die falsch positiven Ereignisse lagen nicht über 3.
Bei 17 der 18 Probanden wurden zytokeratin - positive Zellen nachgewiesen. Die Anzahl der bei Tumorpatienten nachgewiesenen zytokeratin - positiven Zellen betrug im Mittelwert 3,5 +/- 4,0 und bewegte sich in einem Bereich von 0 bis 13.
Das Sorten von zytokeratin - positiven Zellen und die anschließende immunozytochemische Analyse sowie die Mehrfachmarkierung konnten demonstrieren, daß zytokeratin - positive Zellen als Tumorzellen einzustufen sind.
Die Methodenkombination MACS und Durchflußzytometrie ist zur Detektion und zur weiteren Charakterisierung von epithelialen Zellen geeignet.

Flow Cytometric Analyses of CD34-Positive Cells in Peripheral Blood Stem Cell Concentrates: Semi-Automation by Test Kit and Software Support

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The measurement of CD34-positive cells is the most important step in quality control of peripheral blood stem cell (PBSC) apheresis products. For this purpose, flow cytometry is applied. Recently, a new test kit for enumeration of CD34-positive cells (ProCOUNT [PC], Becton Dickinson Immunocytometry Systems [BDIS], San Jose, USA) in combination with software-support by semi-automation for data acquisition and analysis has been introduced. In this study, an evaluation of the kit and software was performed. 90 samples obtained from PBSC apheresis concentrates from 39 patients with hemato-oncological diseases (e.g. NHL, breast cancer) were analyzed by flow cytometry. Aliquots were pipetted into ready-to-use tubes (TruCOUNT, BDIS), and incubated with antibodies (CD34, CD45) and a nucleic acid dye (FL-1). Hereafter, a lyse no-wash procedure was performed. For data acquisition and analysis, ProCOUNT software was used. For data comparison, parallelwise, a second measurement was performed using the German reference protocol (GRP) for CD34-analysis (Infusionsther Transfusionsmed 1996; 23: 1-24). Initial correlation of PC analysis with the GRP was r=0.92 (CD34+/product X 10E6). In 21/90 PC analyses (23%) a warning of the PC software occurred. Following the recommendation for manual re-evaluation with CellQUEST software (BDIS), a correlation of r=0.97 compared to the GRP was obtained. We conclude that the introduction of this kit, capable for CD34-cell quantitation, and the software for semi-automated data acquistion and analysis, represents a promising approach and progress for CD34-cell measurements. However, the occurrence of software warnings has still to be reduced, and reviewing the plots andanalysis results by experienced staff is still mandatory.

Quantitative Digitale Multiparameter Fluoreszenz-Mikroskopie und Durchflußzytometrie: Ein Methodenvergleich zum Separationvermögen Di- und Triploider Lachserytrozyten sowie der Bestimmung Spontaner Apoptosisraten in Frisch Isolierten Mäusethymozyten.

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Hintergrund und Fragestellung: Mikroskopie und Durchflusszytometrie (DZ) wurden bislang meist ergänzend bei der Untersuchung zellulärer Merkmale eingesetzt. Diente die DZ hauptsächlich dazu, eine Zellpopulation statistisch-quantitativ zu erfassen, war die Bestimmung komplexerer morphologischer Merkmale oder subzellulärer Charakteristika (konfokal-) mikroskopischen Einzelzellbeobachtungen vorbehalten. Der Einsatz digitaler Bildverarbeitung ermöglicht es uns, DZ Messungen teilweise zu ersetzen um schnell und direkt statistische Aussagen über eine unter dem Mikroskop beobachtete Zellpopulation zu erhalten. Können Informationen aus Fluoreszenzmessungen gar durch eine Kombination morphologische Parameter ersetzt werden, ermöglicht uns dies Zellen mit einem Minimum an äußeren Einflüssen zu beobachten oder simultan weitere Fluoreszenzfarbstoffe einzusetzen.

Material und Methodik: Die interaktiven Programmsegmente von 'Cell_Calc_2000' wurden in IDL 5.0.2 geschrieben und beinhalten:

1) Routinen zur Direktkorrektur inhomogener Objektausleuchtungen.
2) Bibliotheken isotroper und anisotroper Segmentationsfilter zur Objektdefinition.
3) Eine Bibliothek mathematisch morphologischer Parameter zur Klassifizierung von Objektformen und -texturen, sowie Möglichkeiten der Aufnahme intrazellulärer Leuchintensitäten, -dichten und -verteilungen bei unterschiedlichen Wellenlängen.
4) Eine statistische Analyseroutine zur Transformation des Multiparametersets auf Hautachsen um Trennungs-eigenschaften auf einem möglichst niedrigdimensionalen optimieren Parameterraum zu erhalten.

Die mikroskopischen Messungen der Apoptosisrate frisch isolierter Thymuszellen (RK-Mäuse) wurden eben-so wie die Messungen des DNA-Gehaltes von di- und triploiden Lachserytrocyten mit einem Leiz DIALUX 20 EB durchgeführt. Die Zellen wurden mit den DNA interkalierenden Fluorophoren Acridine Orange (AO), Propidium Iodid (PI), und DAPI, oder aber mit Azur-Eosin-Methylenblaulösung (Giemsas, MERCK) angefärbt. DZ Messungen wurden mit AO durchgeführt. Vorwärts- und 90°-Streulicht des 488nm Lasers wurden neben den Fluoreszenz-intensitäten gemessen.

Ergebnisse und Diskussion: Die Bestimmung des DNA Gehaltes von di- und triploiden Lachserytrocyten ergaben bei vergleichenden mikroskopischen und DZ Messungen annähernd gleichwertige Ergebnisse. Die Informationen aus den Fluoreszenzmessungen konnten durch eine Kombination morphologischer Parameter gleichwertig ersetzt werden. Die Messungen können nun schnell und präzise mit einem einfachen Durchlichtmikroskop durchgeführt werden.

Bei den Messungen der spontanen Apoptosisrate von Thymocyten konnten die DZ Daten ebenfalls reproduziert werden. Zusätzlich markierten die mikroskopischen Daten die bei apoptotischen Thymocyten charakteristische frühe Pyknose, welche mit den DZ Daten nicht identifiziert werden konnte. Für die mikroskopischen Messungen spricht weiter die Möglichkeit, selektiv seltene bzw. schnell stattfindende Ereignisse mit einer XY Position der betreffenden Zellen zu verknüpfen und Substatistiken mit höheren Bildauflösungen zu erstellen.

A New Flow Cytometric Approach for BRDU Detection and Simultaneous Immunophenotyping

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The use of 5-bromo-2-deoxyuridine (BrdU), a thymidine analog, is a unique tool in static and dynamic cell cycle analysis. Its immunological detection with specific monoclonal antibodies allows an easy and accurate determination of DNA synthesizing cells (S-Phase-cells), but requires the generation of single-stranded DNA to expose the epitope. Therefore, conventional methods of producing single stranded DNA by heat or acid treatments often destroy simultaneous immunophenotyping. Over the last decade several enzymatic techniques have been developed that digest DNA such as DNAse I and exonuclease III or label UV-induced single strand breaks(SBIP).

However, lack of sensitivity, overdigestion and artifacts (DNAse), cell loss and tremendous clumping (exonuclease) or the threat of detecting apoptotic events (SBIP) have all been described.

These circumstances encouraged us to develop an enzyme-free approach.

We developed a new zinc based non-crosslinking fixation that allows the extraction of DNA associated proteins with a high-salt solution. The DNA is then accessible to a very mild and short microwave denaturation keeping PE-conjugated surfacemarkers intact. The method has been applied to several cell lines and has proved to be sensitive, reliable and correlate well with the S-phase obtained with acid denaturation.

GENETISCHE VERÄNDERUNGEN VON FRÜHEN OBERFLÄCHLICHEN HARNBLASENKARZINOMEN NACH LASERUNTERSTÜTZTER ODER MANUELLER MIKRODISSEKTION.

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Hintergrund: Die Aufdeckung genetischer Veränderungen vor allem in frühen Harnblasenkarzinomen könnte bei der Beantwortung der Frage helfen, ob das Harnblasenkarzinom eine klonale Erkrankung ist oder sich mehrere maligne Klone gleichzeitig im Sinne einer Feldkanzerisierung entwickeln.
Ziel: Einfache urotheliale Hyperplasien und frühe oberflächliche papilläre Urothelkarzinome, die im Rahmen einer Studie zur photodynamischen Diagnostik des Harnblasenkarzinoms mit 5-Aminolävulinsäure biopsiert wurden, wurden mittels Zweifarben- Fluoreszens in situ- Hybridisierung (FISH) auf die Häufigkeit einer Aneuploidie und von Deletionen der Chromosomen 9 und 17 untersucht.
Methoden: Das Urothel wurde von 20µm- Gefrierschnitten entweder manuell unter dem Invertmikroskop oder mittels lasergestützter Mikrodissektion (PALM) disseziert. Untersucht wurden insgesamt 84 Biopsien (14 einfache Urothelhyperplasien und 70 pTaG1-2- Tumoren) von 22 Patienten. Alle mikrodissezierten Proben wiesen einen Tumorzellanteil von mindestens 90% auf. FISH wurde mit einer Zweifarbentechnik mit biotinylierten Centromerproben für die Chromosomen 9 und 17 und Digoxygenin-markierten genspezifischen P1- Proben für FACC (Chromosom 9q22), CDKI2 (Chromosom 9p21) and p53 (Chromosom 17p) durchgeführt. Signale von mindestens 60 Zellen wurden ausgewertet. Eine Deletion lag vor, wenn mehr als 40% der Zellkerne entweder eine Monosomie oder weniger genspezifische als centromerische Signale aufwiesen.
Ergebnisse: Deletionen des Chromosoms 9 wurden in 70 der 84 untersuchten Proben nachgewiesen (83%). Urotheliale Hyperplasien wiesen mit 71% eine den papillären Tumoren vergleichbare Häufigkeit von Chromosom 9- Verän-derungen. Im Gegensatz dazu konnten p53- Deletionen nur in einer Hyperplasie und 5 papillären Tumoren nachgewiesen werden (7%). Während in der Mehrzahl der Patienten multiple Tumoren die identischen genetischen Veränderungen an den 3 untersuchten Genloci aufwiesen, fanden sich in 4 Patienten unterschiedliche genetische Veränderungen in multiplen Tumoren. Sowohl in Hyperplasien als auch in papillären Tumoren waren Deletionen im Bereich von 9p21 die häufigste nachgewiesene Veränderung, in 15 Proben ohne Vorliegen einer 9q- Deletion. Trisomien deruntersuchten Gene begleiteten oft die frühen Chromosom 9 Deletionen.
Schlußfolgerungen: Das Harnblasenkarzinom ist in den meisten Fällen klonal. Unsere Daten zeigen jedoch, daß auch mehrere Klone in Patienten mit Harnblasenkarzinomen vorliegen können. Deletionen von 9p21 sind die frühesten genetischen Veränderungen. Die präzise Mikrodissektion der Tumorzellen gibt die Möglichkeit, frühe und häufig auch kleine Urothelläsionen zu untersuchen und ist für die sichere Aufdeckung von genetischen Veränderungen in diesen Läsionen unbedingt notwendig. Ein Teil der einfachen Urothelhyperplasien war schon polysom und zeigte Deletionen des Chromosoms 9. Damit sind Hyperplasien zum Teil mögliche Vorstadien von papillären Harnblasenkarzinomen.

POPULATION DYNAMICS OF ACINETOBACTER CALCOACETICUS 69-V AND RALSTONIA EUTROPHA JMP134 IN CHEMOSTAT EXPERIMENTS

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Population dynamics of Acinetobacter calcoaceticus 69-V and Ralstonia eutropha JMP134 growing on phenol in a chemostat were studied flow cytometrically by using in situ-hybridization with fluorescently labelled rRNA-targeted oligonucleotide probes and DNA staining. Both strains showed irregularities in their linear correlation between rRNA-content and specific growth rate when the transientstate-technology for continuous cultivation was utilized. These were also monitored in the subpopulation distribution after DNA staining with DAPI. The comparison of the rRNA contents between the investigated bacteria in the same cultivation regime revealed a much higher ribosome content of Ralstonia eutropha JMP134.
In experiments using a mixed culture of both bacterial strains in continuous culture (S_0 = 1 g/l phenol; D = 0.1 h^{-1}) cells of Ralstonia eutropha JMP134 were washed out whereas the population of the substrate competitor Acinetobacter calcoaceticus 69-V increased. The relative distribution of both strains in the mixed culture was simulated using kinetic models by Monod. It became obvious that for every existing concentration of phenol in the chemostat the specific growth rate of Ralstonia eutropha JMP134 was always lower than of Acinetobacter calcoaceticus 69-V.

External Quality Assurance: Determination of Reference Values for the Complete Blood Count

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For determination of reference values of the concentration of blood cells in control blood samples we have set up flow cytometers as national reference instruments based on impedance measurements or laser light scatter. The reference laser flow cytometer allows to differentiate erythrocytes, thrombocytes and leukocytes in dilute whole blood when observing forward light scatter at 632.8 nm and 413.1 nm. Because of the strong absorption of hemoglobin at 413.1 nm, scattering cross sections of red blood cells (RBC) are reduced substantially and hence leukocytes can be discriminated against erythrocytes. When counting white blood cells (WBC) using impedance flow cytometry, lysis of RBC is required. Besides the concentrations of erythrocytes, thrombocytes and leukocytes, we determine reference values for the hematocrit (Hkt) and hemoglobin concentration (Hb) according to DIN 58933-1 and DIN 58931.

Signals, caused by blood cells crossing the sensitive region of the cytometers, average pulse widths of typically 15 µs for the impedance counter and 2 µs for the laser flow cytometer. In order to correct for counting losses due to coincidences, the pulse width of each signal including electronically caused dead times are summed over all events. The ratio of the integrated dead time and the measuring time, i.e. the parameter of coincidence, is used for the correction of coincidence losses assuming a Poisson distribution of the distances between successive blood cells.

The density of each control blood sample was measured prior to its dilution, which was determined gravimetrically. The volume of the dilute blood sample used for counting blood cells was determined by means of a sensitive balance or a syringe calibrated gravimetrically.

Typically, the variation coefficients (single standard deviation) for reference values amount to 1 % for RBC, 3 % for thrombocytes and WBC, 0.3 % for Hkt and for the concentration of Hb. A comparison of reference values and median values, derived from results of all (1000) laboratories participating in interlaboratory surveys, will be presented.

Flow Cytometric Determination of Volumes of Sphered Erythrocytes by Angular-Resolved Light Scatter

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We have measured angular-resolved light scatter of single blood cells, i.e. native and sphered erythrocytes, thrombocytes, granulocytes, monocytes, and lymphocytes. Individual blood cells of a whole blood sample were preselected observing simultanously integrated forward light scatter at 632.8 nm and 413.1 nm as well as orthogonal light scatter at 413.1 nm in a flow cytometer. About 25 µm downstream the blood cells intersected an Ar^+ laser beam. Angular resolved light scatter of a blood cell crossing the focus of the Ar^+ laser beam was recorded by opening the intensifier of a CCD-camera, provided their integrated light scatter fell within a selected region of the corresponding (integrated) scatter diagram. For observation, a 32x / 0.6 microscope objective with its axis oriented orthogonal with respect to three collinearly propagating laser beams and the direction of the sample flow was used, allowing to detect light within a range of polar angles $63° \leq \theta \leq 117°$ at the azimuth angle $\varphi = 90°$.

Angular-resolved light scatter of sphered red blood cells was analyzed by comparing measured angular distributions, i.e. normalized differential cross sections, with differential scattering cross sections calculated by Mie theory. The volume of each sphered blood cell was determined by means of a least squares fit or by calculating the correlation function between theoretical and measured normalized differential cross sections. It should be noted, that several local minima (maxima) exist for volumes ranging between 60 fl and 140 fl. In order to obtain the correct volume, absolute differential cross sections have to be measured with an accuracy of at least 20 %. Combining integral and angular resolved light scatter, volumes of sphered red blood cells can be determined with an accuracy of about 1%.

First results on measured volume distributions of erythrocytes will be presented. Furthermore we report on experiments with polystyrene microspheres to validate the method and on the influence of the index of refraction.

ISH Analyses of Tissue Sections. Novel ISH Detection Systems to Study Chromosomal Imbalances, Gene Amplifications and Genetic Heterogeneity

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There are major advantages of ISH on tissue sections over ISH on isolated tumor cells. 1) Heterogeneity of tumor cell areas with chromosome aberrations can be recognized in the tissue sections and can be correlated with histologic appearance. 2) No selection of cells occurs as a result of the isolation procedure and 3) small lesions up to 10 - 20 cells can be evaluated. To overcome misinterpretation of ISH signals, which are the result of nuclear truncation, we performed double-target ISH and determined the signal ratio between both probes. This approach allows the detection of aberrations such as monosomies, trisomies and amplifications within one single tissue section. The prerequisites for signal ratio imaging are preservation of the nuclear morphology during the entire ISH procedure, ISH pretreatment protocols which are independent from variations in routine processing of patient material (e.g. fixation conditions) and strong ISH signals. For the first two conditions several chemical retrieval steps were combined with pepsin to pretreat the formalin fixed paraffin embedded tissue. In order to increase the ISH detection sensitivity the catalyzed reporter deposition (CARD) method was included. This method is based on the deposition of haptenized or fluorochrome tyramide molecules in the vicinity of hybridized probes catalyzed by the enzyme horseradish peroxidase. Biotin, digoxigenin, dinitrophenyl as well as fluorescein, rhodamin and coumarin labeled tyramides can be applied in single, double and triple color ISH reactions. Compared to standard fluorescence and chromogenic staining procedures, the application of the CARD system strongly increases the signal intensity. This facilitates amongst others the detection of low-copy nucleic acids, visualization of gene amplifications, and the manual as well as automated scoring at lower microscopic magnification. Furthermore fluorochrome labeled tyramides reduced the number of immunocytochemical steps since no repeated rounds of incubations are needed. Examples will be given how these methods were used to study CIS of the bladder, breast cancer, head and neck tumors using repeat sequence probes as well as locus specific probes. The ISH analysis of tissue sections is an essential step to gap data obtained by e.g. microsatellite analyses and CGH on the one hand and the ploidy/aneusomy changes on the other hand. The latter ploidy changes and chromosomal imbalances strongly influence the interpretation of these data. In addition the ISH analysis links chromosomal aberrations to histologically classified areas including e.g. transitions from normal to hyperplasia/dysplasia, changes in nuclear morphology or to processes as invasion. In many cases this involves the analysis of a limited number of cells.

Quantitative Measurement of Gap Junctional Coupling by Pre-Loading a Cell Population

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Quantification of intercellular communication via gap junctions is difficult to accomplish when individual cells have to be impaled with an electrode for dye or current injection. Results obtained by this method vary with the experience of the investigator. We propose a way out of this dilemma by combining the pre-loading technique of Goldberg et al. [1] with flow cytometry where a large number of cells can be examined in a single experiment. Suspended cells are loaded with the membrane permeable dye Calcein AM which is cleaved intracellularly by unspecific esterases and is thus trapped inside the cells. Gap junctions, however, are permeable for this fluorescent dye. This can be observed when pre-loaded cells are added to a sparse monolayer culture and cell-cell contact is established within 40 - 60 minutes. When one of these two cell populations is additionally stained with a membrane resident dye (DiI, DiA) different cell populations can be separated not only microscopically but also by flow cytometry. We have used this technique to re-investigate homo- and heterotypical coupling in connexin-transfected HeLa cells.

[1] G. S. Goldberg, J. F. Bechberger, and C. C. G. Naus: A Pre-Loading Method of Evaluating Gap Junctional Communication by Fluorescent Dye Transfer. BioTechniques 18, 3, 490-497 (1995)

In Vivo Visualisierung von Leukozyten/ Endothel Interaktion. Induktion durch Extrakorporale Zirkulation

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Die klinischen Komplikationen der EKZ (z.B. bei kardiopulmonalem Bypass, extrakorporaler Membranoxygenierung oder bei Hämodialyse) werden mit einer Aktivierung von zellulären und humoralen Mechanismen in Verbindung gebracht. Dies kann zu einer systemischen inflammatorischen Reaktion sowie zu einer Dysregulation im Bereich der Mikrozirkulation bis hin zum Organversagen führen. Da zur Untersuchung der Pathophysiologie nur in vitro Methoden existieren, wurde von uns ein Modell zur Evaluierung der Effekte von EKZ auf die Mikrozirkulation entwickelt.
Das Modell der Hamsterrückenkammer erlaubt die intravitalmikroskopische Untersuchung der Mikrozirkulation von Haut und Skelettmuskulatur am wachen Versuchtier. In Pentobarbitalanästhesie wurden eine Titanium-Beobachtungskammer sowie arterielle und venöse Dauerkatheter implantiert. Nach Applikation von Rhodamin 6G zur Leukozytendarstellung sowie von Heparin (300 IE/kg KG i.v.) wurde mit Hilfe einer Mikrorollerpumpe sowie eines Silastikschlauches Blut zwischen der ACC und der VJI zirkuliert.
Die isovolämische EKZ für die Dauer von 20 min. führte zu einer Zunahme der rollenden (10 + 4% auf 38 + 20 %) und adhärenten Leukozyten (18 + 16/mm^2 auf 215 + 145/mm^2) in postkapillären Venolen (Mittelwert ± Std.; n = 7, one way ANOVA: * $p < 0.05$). Als Kontrolle und um z.B. Hämodilutionseffekte auszuschließen, wurde außerdem eine EKZ für die Dauer von 10 und 2 min durchgeführt. Es zeigte sich, daß die Zunahme an adhärenten Leukozyten abhängig war von der Dauer der EKZ. Die funktionelle Kapillardichte wurde nicht beeinträchtigt, arterieller Blutdruck sowie Herzfrequenz waren stabil.
Unsere Experimente zeigen in vivo, daß Blutkontakt zu einer FremdoberRäche Leukozyten/Endothel Interaktionen induziert. Dies kann als Zeichen einer systemischen Entzündungsreaktion gewertet werden. Die neue Anwendung des Hamstermodells soll dazu beitragen, den zugrundeliegenden Pathomechanismus zu untersuchen sowie therapeutisch/prophylaktische Strategien zu entwickeln, um die mit der EKZ verbundenen Probleme zu lösen

Influence of Lipid Metabolism on Surface Receptor Expression and Related Signal Transduction in Human Monocytes

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The LPS-receptor CD14 and the u-PA-receptor CD 87 that play central roles in monocyte activation are anchored in the plasma membrane via a glycosyl phosphatidylinositol residue (GPI). In several cell models GPI-anchored receptors have been shown to be associated with rafts and caveolae, which are cholesterol- and sphingolipid-enriched microdomains of eukaryotic plasma membranes. The simultaneous coassociation of transmembrane receptors has been postulated to be important for GPI-receptor dependent signal transduction. In a recent ex vivo study on patients treated with the HMG-CoA-reductase inhibitor fluvastatin we observed a significant decrease of CD14 expression on monocytes in correlation with the decrease of plasma cholesterol. The goal of this in vitro study was to address whether both the expression of GPI-anchored receptors and the receptor coupled signal transduction on monocytes are modulated in a membrane cholesterol dependent manner.
Endogenous deprivation of cholesterol through incubation with fluvastatin for up to 72 hrs in serum-free M-CSF containing medium induced a dose dependent upregulation of the transmembrane Fcγ-receptor CD16 expression while a decrease of CD14 expression was observed only at higher fluvastatin concentrations. Exogenous depletion of cholesterol and sphingomyelin performed by incubation of monocytes in the presence of cholesterol oxidase or sphingomyelinase for up to 2 hours in contrast induced a dose and time dependent decrease in CD14 expression. Only the latter treatment interestingly also caused impairment of [Ca2+]i response to CD14 crosslinking already at a concentration and incubation time not affecting CD14 expression.
These results show that membrane cholesterol is an important modulator of signal transduction via GPI-anchored molecules. Fluvastatin thus reveals to have a yet uncharacterized complex immunomodulatory effect on monocyte differentiation which may be related to the antiatherogenic benefits of the drug.

Cytotoxic but not Cytostatic Effects of Doxorubicin Depend on P53 Status in Leukemia Cell Lines

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Most biologically active molecules used in chemotherapy, including anthracyclines, affect cell cycle progression and induce cell death by apoptosis. Both effects have been suggested to depend on functional activity of p53 protein which, when overexpressed, induces cell cycle arrest and apoptosis in G0/G1 phase. However, anthracyclines are known to have multiple molecular targets in malignant cells including regulators of G2/M cell cycle phases. To address relationships between p53-dependent and -independent pathways induced by chemotherapeutic agents and their cell cycle specificity we investigated dose dependent effects of doxorubicin (0.01-5.0 µM) in human leukemia cell lines with different p53 status. To this end, we applied the recently developed flow cytometric method based on Annexin V staining of apoptotic cells and propidium iodide staining of nuclear DNA, which permits cell cycle analysis in samples undergoing apoptosis. Assessment of apoptosis demonstrated high sensitivity of cells with wild type p53 (wt/wt-p53: MOLT-3, MOLT-16) which showed 30-90% of apoptosis at 0.2µM of doxorubicin, while cell lines with heterozygous p53 mutations (wt/mut-p53: Jurkat, P12/Ichikawa, KE-37) and negative for p53 (null-p53: K562, HL60) were resistant to these doxorubicin concentrations. Analysis of cell cycle effects in parallel to apoptosis disclosed that at 0.2 ,uM doxorubicin induced cell cycle arrest in G2/M phase. Interestingly, the G2/M arrest was observed in all cell lines independently of their p53-status. At higher doxorubicin concentrations (1-5 µM), G0/G1 cell cycLe arrest and p53independent apoptosis could be observed in wt/mut-p53 and nullp53 cell lines. Taken together, our data suggest that the cell cycle checkpoint in G2/M is the most sensitive one to the treatment with doxorubicin Moreover, in contrast to apoptosis, the cell cycle arrest in G2/M does not depend on p53 status of leukemia cells.

High-Sensitivity Detection of Intracellular Antigens by Flowcytometry Using the Tyramin Signal Amplification (TSA)

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Intracellular immunofluorescence has become a powerful tool for analysis of secreted molecules *en route* to secretion or cytoplasmic antigens on the single cell level. However the quantitative detection of cytokine producing cells is sometimes hampered by the fact that only low numbers of molecules are expressed per cell. The Tyramin-Signal-Amplification (TSA) uses an enzymatic signal amplification step in combination with conventional immunofluorescent labelling. So far it has only been applied to immunhistochemistry and immunfluorescence of tissue sections. Here we show the application of the TSA-method for staining of cells in suspension, which allows the detection of weakly expressed intracellular antigens by flow cytometry. Conditions were established to circumvent cross reactivity and unspecific labelling by the TSA-method. Looking at cytokines as model antigens the signal intensity as well as the detection limit could be improved up to ten-fold. TSA-staining can easily be combined with conventional labelling methods and, using various substrates, also TSA-multicolour staining could be performed.

Protoporphyrin-IX-Metabolismus in Urothelzellen als Grundlage der differentiellen Fluoreszenz in der photodynamischen Diagnostik von Harnblasentumoren

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ZIEL: Die Instillation von 5-Aminolävulinsäure (ALA), einer Vorstufe im Häm-Biosyntheseweg, in die Harnblase führt zu einer erhöhten Konzentration von Protoporphyrin IX (PPIX) im Tumorgewebe. Diese selektive PPIX-Akkumulation wird zur sogenannten photodynamischen Diagnostik von Tumoren und Tumorvorstufen eingesetzt. Nach Anregung mit blauem Licht können die neoplastischen Areale anhand ihrer ALA-induzierten roten PPIX-Fluoreszenz erkannt werden. Um die grundlegenden Mechanismen dieser differentiellen PPIX-Akkumulation zu untersuchen, wurden PPIX-Konzentration, Ferro-chelataseaktivität, Eisengehalt und Transferrinrezeptordichte (CD71) an humanen urothelialen Zellinien untersucht.
MATERIAL & METHODEN: Zwei Harnblasenkarzinomzellinien (J82 und RT4), eine Urothelzellinie (UROtsa) und eine Fibroblastenlinie (N1) wurden im exponentiellen und Plateau-Wachstumszustand untersucht. Nach Inkubation mit ALA (0-200 µg/ml, 3 h) wurde die zelluläre PPIX-Fluoreszenz durchflußzytometrisch gemessen. Parallel dazu wurde nach Extraktion eine fluoreszenzspektrometrische Bestimmung der PPIX-Konzentration pro Proteinmenge durchgeführt. Zur Bestimmung der Ferrochelataseaktivität wurden die Zellen zusätzlich zu ALA (200 µg/ml) mit dem Eisenchelator Desferrioxamin (DEF; 0-750 µM) inkubiert, und der DEF-induzierte PPIX-Anstieg am Durchflußzytometer quantifiziert. Der intrazelluläre Eisengehalt wurde photometrisch über die Bildung eines Eisen-Ferrozin-Komplexes nach Homogenisierung bestimmt. Die Messung der Transferrinrezeptordichte erfolgte durchflußzytometrisch nach indirekter Immunfluoreszenzmarkierung.
ERGEBNISSE: Zellen mit hoher PPIX-Konzentration besitzen geringe Ferrochelataseaktivität oder geringen Eisengehalt. Zellen mit geringer PPIX-Menge haben hohe Ferrochelataseaktivität oder hohen Eisengehalt. Insbesonders die Zellinie J82 mit der höchsten PPIX-Akkumulation, abgeleitet von einem invasiven, niedrig differenzierten Urothelkarzinom, zeichnet sich sowohl durch eine niedrige Ferrochelataseaktivität, als auch durch einen geringen Eisengehalt aus. Der Transferrinrezeptor ist für alle vier Zellinien ein Indikator für die Proliferation der Zellen, steht aber in keinem Zusammenhang zu Eisengehalt oder PPIX-Konzentration.
SCHLUSSFOLGERUNGEN: Das verwendete In-vitro-Modell spiegelt die differentielle Fluoreszenz zwischen Tumor und Urothel/Bindegewebe der In-vivo-Situation bei Zystoskopie nach ALA-Instillation wieder. Die Untersuchung der Mechanismen, die zur selektiven PPIX-Akkumulation führen, bildet die Grundlage für eine Optimierung dieses diagnostischen Verfahrens und muß durch die Analyse der ALA-Aufnahme und des Enzyms Porphobilinogen-deaminase erweitert werden.

Mit Unterstützung durch die Mildred-Scheel-Stiftung (70-2200-Ba-2).

MicroFlow 6.0 - das Leipziger Cytometersystem für die Biotechnologie

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Die Flow Cytometry hat sich zu einer leistungsfähigen Methode für die Quantifizierung der mikrobiellen Populationsdynamik in biotechnischen Prozessen entwickelt.
Auf Grund der im Vergleich zu medizinischen Meßobjekten geringen Größe der zu messenden Partikel (Hefen und Bakterien) mit geringen Mengen fluoreszenzmarkierbarer Inhaltsstoffe sind die Anforderungen an ein Flow Cytometer sehr hoch. So hat ein modulares Cytometersystem, das auf spezielle Fragestellungen zugeschnitten ist, Vorteile gegenüber Geräten, die für die Anwendung in der Medizin entwickelt wurden.
Anfang der 80er Jahre wurde in Leipzig begonnen, ein Cytometer für die Untersuchung von Meßobjekten der Biotechnologie, zunächst Hefen, aufzubauen.
In der modernen Biotechnologie spielen Bakterien eine immer größere Rolle. Das Cytometersystem wurde und wird erweitert, so daß damit auch Zustandsverteilungen von Prokaryoten bestimmt werden können.
Das Cytometer in der jetzigen Ausbaustufe wird vorgestellt. Die wesentlichen Komponenten sind:

Optik : mit der Schnittstelle Photomultiplier zur Meßhardware
Meßhardware (PC-extern): mit der Schnittstelle PC – Einsteckkarte zur Meßsoftware

Meßsoftware:
Die **Optik** umfaßt den Teil des Cytometers von der Vereinzelung der zu messenden Partikel über die Excitation dieser Teilchen bis zur Detektion der Fluoreszenzemissionen und der Streulichtintensitäten.
-Hüllstromkammer: Die hydrodynamische Fokussierung des Probenstromes erfolgt in einer Freistrahlkammer.
-Lichtanregung: Zur Lichtanregung stehen 3 Laser (Argon-Ionenlaser, UV-fähiger Argonionenlaser, HeNe - Laser) zur Verfügung, die auch wahlweise gekoppelt werden können (über Strahlteiler oder umgekehrtes Prisma-Prinzip).
-Lichtdetektion: Die Lichtdetektion erfolgt mit Photomultipliern. Sie erzeugen einstellungs- und partikelabhängig Ströme im Bereich weniger Mikroampere und über weniger als eine Mikrosekunde. Dementsprechend müssen die danach folgenden Strom/Spannungswandler qualitativ hochwertig für den Megaherzbereich ausgelegt und dicht am Photomultiplier aufgebaut werden.

Die **Meßhardware** besteht aus Kanalplatinen und einer Downloadplatine.
-Download: Über eine serielle Schnittstelle werden alle Größen zur Parametrierung der Messung vom PC einem Mikrokontroller in der externen Hardware mitgeteilt, der die Kanalplatinen initialisiert.
-Kanäle: Den programmierbaren Vorverstärkern mit maximal 64-facher Verstärkung folgen Spitzenwerthalter, der die zu messende Größe ermittelt. Der Mikrokontroller führt einen Handshakedialog mit der PC-Einsteckkarte, die die maximal acht Kanalsignalmaxima von den Kanalplatinen übernimmt.

Die **Meßsoftware**, die mit LabWindows/CVI erstellt wurde, realisiert:
- Parametrierung und Speicherung der Meßumgebung
- Online-Visualisierung der gemessenen Daten in 2 oder 3-dimensionalen Histogrammen
- Ablage der Daten im FCS 2.0 – Fileformat.

The Potential Usage of Laser Scan Cytometry in Clinical Diagnostics

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Laser scanning cytometry provides multicolor fluorescence measurements on a slide as well as microscopy images for further detailed morphological investigation. Therefore, the LSC combines the capabilities of flow cytometry with light and fluorescence microscopy techniques.
For clinical diagnostics in pediatric cardiology, the LSC enables to minimize the needed sample volume. As we will show, it is possible to do extensive immunological investigation from a 5µl blood sample only.
Compared to commonly used flow cytometry, the LSC lacks of side scatter dectection. Therefore, fluorescence labelling strategy for the LSC is different. Even forward scatter usage limits the total amount of four to three colors. Despite of this, additional information can be attained by relocating single cells for further microscopcial investigation. Beside visible light, the microscopy performance includes even the UV range, whereby the remaining of a new radiopaque material used for echocardiography in blood successfully was identified.
In a FISH application, an induced genetic defect on murine Y-chromosom was identified via a FITC labelled probe versus PI counter staining.
We yet started to perform our immunological investigation of children undergoing heart surgery via Laser scanning cytometry with promising first results.

Tumor Scanning via NADH Autofluorescence - The Interbolism Detector

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The coenzymes NADH and NADPH play a major role in the cell´s interbolism symbolizing its metabolic activity. Its autofluorescent property are well-known and have already been used for monitoring changes of the interbolisms. The new developed Interbolism Detector was originally developed for tumor diagnostics, but with its sensitivity and performance the detector was also used for therapeutical drug monitoring (TDM) and other biotechnological applications. The Interbolism Detector consists of a N_2-LASER and a glasfiber sensor small in diameter (100-800 µm sensor). By time-resolved fluorescence detection in a 2ns gate, the detector is advanteuos in sensitivity and accuracy.
It is well known fact that the interbolic activity of tumor is much higher compared normal and healthy tissue. This can be utilized for scanning the tumor´s location and expansion invasively before and during surgery. We will show several in vitro studies on scanning solide tumors after sugery embedded in normal tissues.
Our data exhibit that the Interbolism Detector is a performing diagnostical instruments which can be used to support invasive ecotomy as well as to localize subcutaneously expanding carcinomas.

Bestimmung des Absoluten DNA-Gehaltes in Pflanzlichen Zellkernen - Vergleich der Ergebnisse von vier Laboratorien

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Die Durchflußzytometrie wird zunehmend zur Bestimmung des nuklearen DNA-Gehaltes von Pflanzen eingesetzt. Ziel des hier beschriebenen gemeinsamen Experimentes war die Untersuchung der Zuverlässigkeit dieser Methode, indem in vier Laboratorien der nukleare DNA-Gehalt von neun Pflanzenarten in einem Bereich von 2C = 0.3 - 30 pg mit interner Standardisierung gemessen wurde.

Die beteiligten Laboratorien verwendeten unterschiedliche Methoden zur Kernisolation, Puffer und Analysengeräte. Die Ergebnisse mit interkalierenden Farbstoffen (Ethidium-Bromid, Propidium-Jodid) stimmten sehr gut mit denen der Feulgen-Mikrophotometrie und untereinander überein. Dagegen zeigten die mit DAPI erhaltenen Resultate deutliche Abweichungen auf Grund der AT-Spezifität.

Die Ergebnisse zeigen, daß die Durchflußzytometrie mit interkalierenden Farbstoffen eine zuverlässige Methode zur Bestimmung der nuklearen Genomgröße in Pflanzen darstellt.

Die Untersuchungen wurden finanziell unterstützt durch den Österreichischen Fonds zur Förderung der Wissenschaftlichen Forschung, Projekt P9593-BIO und Grant Nr. 521/96/K117 der Grant Agency der Tschechischen Republik.

Neue Perspektiven für die Immunologische Zytometrie

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Die dringend notwendige Entwicklung gezielterer und nebenwirkungsärmerer Behandlungsverfahren für Erkrankungen des Immunsystems wird durch in ihrer Aussagekraft sehr begrenzte diagnostische Möglichkeiten stark verlangsamt. Immunologische Diagnostik wird derzeit auf der Ebene von Proteinen durchgeführt. Trotz der Perfektionierung beim Einsatz monoklonaler Antikörper in zytofluorometrischen und solid-phase immuno-assays muß man erkennen, daß die Sensitivität dieses Zuganges limitiert ist. Probenmengen, die man benötigt, um qualifizierte Analysen in notwendigem Umfang durchzuführen, sind in der Regel nur aus dem peripheren Blut des Menschen zu erhalten - einem Kompartiment, in dem sich aber nur die wenigsten der o.a. Erkrankungen abspielen und sich deswegen auch dort nicht ausdrücken.

Eine neue Perspektive bieten quantitative analytische Verfahren auf der Ebene der Genexpression mittels PCR. Wegen der hier möglichen exponentiellen Amplifikation von Signalen ist es möglich, aus minimalen Probenvolumina multiple Analysen durchzuführen. Die Kombination der PCR mit einer zeitgemäßen apparativen Ausstattung und leistungsfähiger Datenverarbeitung eröffnet erstmals die Chance, umfangreiche immunologische Diagnostik aus vom individuellen Krankheitsort entnommenen Gewebsproben vorzunehmen. Wir erwarten von diesem Zugang, krankheitsspezifische Profile zu identifizieren und therapeutische Interventionen anhand deren Veränderungen zu steuern. Gleichzeitig sollten diese Ansätze es erlauben, durch Erfassung relevanter Parameter an der "Endstrecke" Immunsystem, immunsuppressive Therapie auf die individuellen Gegebenheiten eines jeden Patienten einzustellen.

THROMBOGENITY OF CORONARY STENTS: AN *IN VITRO* ASSAY

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One of the most frequent complications after PTCA and stent implantation, are acute and subacute restenoses. Clinical studies indicate that material and structure of coronary stents strongly correlate with the incidence of arising complications. Therefore, we developed an assay for testing the platelets activation in vitro via flow cytometry dependent on used stents.
Distinguished by material or design, various stents (8) were investigated. Only blood from drug free, healthy, male volunteers in the age of 20 to 35 years was used. After vein punction, blood drops were directed through a silicon coated glass funnel ending in a silicon tube in which a stent was inserted. Blood was collected in a citrate tube for further investigation. Similar construction without stent was used as control. Activation of platelets receptors (CD41a, CD42b and CD62p) and platelet-platelet aggregates were determined in dual laser flow cytometry via no-wash method (with whole blood). We used CD14, CD45 and CD41 labeling, for identification of leukocyte-platelet interaction. Most distinct differences were seen for monocyte-platelets aggregates and granulocyte-platelet aggregates.
In fact, differences due to the set were significant, i.e. heparin or gold stents induce only minor platelet activation compared to stents made of steel. Flow cytometry is capable for testing biocompability of stents in our experience. In the next future, experiments are planned to draw conclusions about the relevance of thrombogenety regarding restenosis after PTCA with stent-implantation.

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NEW FLOW CYTOMETRIC ASSAYS FOR CLINICAL ANALYSIS OF LEUKOCYTE FUNCTION: NATURAL KILLER CELL ACTIVITY AND BASOPHIL DEGRANULATION

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Flow cytometry is now widely used in clinical hematology and immunology. We have extended its use in immunophenotyping of lymphocyte subsets for the diagnosis of immunodeficiencies and hematological disorders by analysing the functional capacity of leukocytes. Phagocytosis and oxidative burst have already been proven valuable in the diagnosis and monitoring of immunodeficiencies and sepsis. A prerequisite for a clinical application is a simple, robust and validated test system.
NK activity is important for the removal of virus infected and tumor cells. In order to simplify the the test, we used frozen and fluorescent, ready to use target cells. By this way it was possible to reproducibly monitor a clinical study and to diagnose Chediak-Higashi-Syndrome, a disease where the cytotoxic granules of NK cells are missing.
A new test for basophil degranulation was developed to improve the diagnosis of allergies. The classical test for specific IgE against a certain allergen (RAST) requires coupling of allergens and haptens to artificial carriers and matrices. Some allergens are not available or inactivated by the chemical process. Alternative methods like histamine release assay or CAST ELISA are cumbersome and time consuming. We developed a simple assay that detects the de novo expression of gp55 on the cell surface of basophilic granulocytes upon degranulation by immunofluorescence. The assay works in whole blood and requires two hours. It allowed us to demonstrate the sensitisation of many clinical employees to latex induced by powdered gloves.
HIV and drug induced apoptosis, early lymphocyte activation and cytokine production are other valuable functional cytometric assays.
In summary, these assays of leukocyte function largely extend the clinical use of flow cytometry while the analysis of lymphocyte subsets has a limited application.

Comparison of HLA-DR4 Typing by Flow Cytometry and Polymerase Chain Reaction Using Sequence Specific Primers

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Patients with rheumatoid arthritis (RA) show a higher frequency of HLA-DR4 (50 %) than healthy controls (17 %). The onset of the alleles DRB1*0401 and DRB1*0404 are regarded as markers for a severe prognosis of RA. Particular subtypes of HLA-DR1, 4, 10 bear a shared epitope (SE) at AA position 70-74 of the β1 chain. This epitope is present in 80-90% of patients with RA.

The aim of our study was to check the accuracy and reliability of a flow cytometric (FC) method (Medac, Germany) that allows phenotyping of HLA-DR4, SE, and of the gene products of the alleles DRB1*0401 and DRB1*0404 with an indirect immunolabeling technique compared with a reliable PCR method serving as reference.

201 FC typing results from patients with rheumatoid arthritis and other inflammatory rheumatic diseases (reactive arthritis, ankylosing spondylitis, psoriatic arthritis and mixed connective tissue diseases) were compared with those obtained by a PCR-SSP test (Deutsche Dynal GmbH, Germany) allowing a low resolution HLA-DR typing and a high resolution typing of HLA-DR4 subtypes.

In the PCR-SSP 109 patients were positive for HLA-DR4 (54.23%), 81 for the SE (36.82%), 61 for HLA-DRB1*0401 (30.35%), and 10 for HLA-DRB1*0404 (4.98%). In comparison to this method the FC test showed false results for DR4 in 4.59%, for the SE in 8.11%, for HLA-DRB1*0401 in 20.40% and for HLA-DRB1*0404 in 2,49% of all of the patients.

The investigation shows that HLA-DR4 typing by FC is useful for rapid typing in order to support the prognosis of RA. Nevertheless, in our hands it not appropriate for the accurate determination of the gene products of the alleles HLA-DRB1*0401 and 0404.

Reduction of Infection Risk for Creutzfeldt-Jakob Disease (CJD) by Removal of B Lymphocytes by Inline Filtration. Evaluation of the Efficiency of Polyester Filters by Flow Cytometric Counting.

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Prevention of transfusion related transmission of infectious prions is an emerging concern. Animal models with B cell deficient mice showed that the presence of B cells plays a central role in transmissible spongiform encephalopathies (Klein MA. et al., Nature 390(6661), 587, 1997). A potential risk reduction of CJD infection might be achieved by inline filtration of blood due to removal of B cells. New generations of polyester filters eliminate the majority of white blood cells (WBC) including B cells in a magnitude of 4 logarithmic decades.

The capacity of polyester filters to reduce B cells was investigated in whole blood (EDTA), RBCC or FFP of 30 donors by flow cytometry (FC). T and B cells were counted using the Tritest-Trucount reagents CD45-PerCP, CD3-FITC and CD19-PE (Becton & Dickinson, USA). The Trucount tubes allow the determination of the exact acquisition volume and consequently the calculation of the absolute numbers of labeled cells. In a first measurement both T and B cells were counted in whole blood, in RBCC and FFP. Since the number of B cells is usually below the detection limit of the FC method in RBCC and FFP, the mononuclear cells of 50 ml of each blood component were concentrated using a Ficoll-Hypaque separation medium (Pharmacia, Sweden). In this fraction the ratio of T and B cells was again determined whereby their exact number could be interpolated from the total number of T plus B cells of the first measurement.

In average in the whole blood of the donors $1.33 \times 10^8 \pm 6.36 \times 10^7$, in FFP $9.97 \times 10^3 \pm 2.08 \times 10^4$ and in FRCC $3.68 \times 10^3 \pm 6.91 \times 10^3$ B cells/unit could be determined. Compared with T cells B cells were retained approximately 4000 times higher in RBCC and 200 times higher in FFP. The results show that reduction of B cells in FFP and RBCC after inline filtration might diminish the risk of a transmission of prions in transfusion recipients.

Phänotypisierung von Disseminierten Tumorzellen im Knochenmark Mittels Cytokeratin 8/9/18 und uPA-Rezeptor-Antikörper durch Konfokale Laserscanmikroskopie (CLSM)

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Immunhistochemisch lassen sich bei über einem Drittel aller Patienten mit Brust- und Kolonkarzinomen vereinzelte Tumorzellen im Knochenmark nachweisen. Untersuchungen an Magenkarzinom-patienten haben gezeigt, daß die Detektion von uPA-Rezeptor-positiven Tumorzellen im Knochenmark mit einem hohen Rezidivrisiko verbunden ist. Patientinnen mit agressiven Tumoren lassen sich identifizieren und einer risikoadaptierten, individualisierten Therapie zuführen.

Fragestellung: Können disseminierte Tumorzellen bei Mammakarzinompatientinnen und Patienten mit gastrointestinalen Malignom-erkrankungen mittels monoklonaler Antikörper gegen Cytokeratin-filamente 8,9 und 18 nachgewiesen werden und lassen sich diese Zellen in bezug auf ihre uPA-Rezeptor-Expression weiter charakteri-sieren?

Methode: Durch CLSM und FACS-Analyse wurden 3 monoklonale Mausantikörper gegen Cytokeratin 8/9118, sowie 6 monoklonale und 2 polyklonale Antikörper gegen den uPA-Rezeptor an Malignomzellen (ZR75, MCF7, OVMZ 6,U 937 und Raji), peripheren Blutlymphozyten (PBL) und Knochenmarkzellen ausgetestet. Ein monoklonaler Mausantikörper gegen Cytokeratin 8/9/18 (A 45 B 3/3) und drei Antikörper gegen uPA-Rezeptor (11 D 7, 111 F 10 und HD 13.1) wurden mit Fluorochromen direktkonjugiert. Doppelmarkierungen an Knochenmarkausstrichen und Cytospinpräparaten von Mamma- und Kolonkarzinompatienten wurden durchgeführt.

Ergebnisse: Mit der Methode des kombinierten Nachweises von Cytokeratin 8/9/18 und uPA-Rezeptor lassen sich Tumorzellen im Knochenmark mit hoher Sensitivität identifizieren und charakterisieren.

Schlußfolgerung: Zur Selektion von Hochrisikopatienten werden Knochenmarkaspirate von Mammakarzinompatientinnen mit Hilfe der neuen Doppelmarkierungstechnik untersucht.

Bestimmung numerischer Aberrationen der Chromosomen 1 und 7 in Zytologischen Präpataen der Cervix Uteri Mittels FISH

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Hintergrund
Numerische chromosomale Aberrationen spielen wahrscheinlich bei der Karzinogenese von Plattenepithelzell-Karzinomen der Cervix uteri eine Rolle. Flowzytometrisch kann die Ploidie sehr gut bestimmt werden, die einzelnen betroffenen Chromosomen können aber nicht identifiziert werden. FISH (Fluoreszenz-in-situ-Hybridisierung) ist eine einfache Methode um numerische Abweichungen einzelner Chromosomen in Interphasenkernen zu bestimmen.

Ziel
Wir erhoben die Häufigkeit von numerische Aberrationen der Chromosomen 1 und 7 in zytologischen Präparaten der Cervix uteri mittels FISH, um einen Zusammenhang von numerischen Abberrationen dieser beiden Chromosmen und der Entwicklung von präinvasiven und invasiven Läsionen zu evaluieren. Weiters sollte die Anwendbarkeit von FISH als mögliche Screeningmethode bei zytologischen Präparaten zur genaueren Identifizierung von präinvasive Läsionen erprobt werden.

Material und Methode
Bis zum jetztigen Zeitpunkt wurden zytologische Smears von 9 CIN III (cervicale intraepitheliale Neoplasie) und als Kontrolle von 8 PAP II mittels FISH evaluiert. Es wurden zentromerische DNA-Sonden für das Chromosom 1 und 7 verwendet. Im Mittel wurden 72 Kerne pro Präparat ausgezählt (Range 27-118 Kerne). Die Anzahl der Signale für Chromosomen 1 und 7 wurde in jedem Kern gezählt und zwischen den beiden Gruppen verglichen.

Resultat
In den Zellkernen der CIN III fanden sich signifikant häufiger drei Signale für das Chromosom 7 als in der Gruppe der PAP II ($p<0.05$). In den Kernen der CIN III Gruppe fanden sich häufiger drei Signale für das Chromosom 1 und vier Signale für die Chromosomen 1 und 7, der Unterschied war aber statistisch nicht signifikant.

Schlußfolgerung
Für das Chromosom 7 findet sich in präinvasiven Cervixläsionen häufig eine Trisomie, die auf eine mögliche Rolle diese Chromosoms bei der Entwicklung von Cervixkarzinomen hinweist. FISH ist eine Methode die zur genaueren Evaluierung von zytologischen Routinepräparaten eingesetzt werden kann.

Onset and Synchronisation of Optically Induced Focal Calcium Oscillations in Syncytia of Cardiac Myocytes

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During enzymatic digestion of heart tissue the temporal automaticity of the entire heart is preserved on the single cell level and appears as oscillation of the cytosolic calcium concentration. Embryo derived heart cells close up, connect via gap junctions and self-organize as a cytosolic continuum enveloped by a single cell membrane. This oligocellular syncytium behaves as a network of weekly coupled oscillators. A two-dimensional muscle-like tissue is reconstituted in cell culture.
During the release of calcium ions with subcellular resolution by photolysis of np-EGTA with a microbeam a cell group undergoes a transition from dormancy to oscillation. The onset of the oscillation and its spreading over the entire cell group can be visualized by real time calcium imaging with the calcium sensitive dye Calcium Green1. This process starts with an exponential decrease of the calcium concentration in the cytosol subsequent to the calcium dependent activity of the sodium/calcium-exchanger. The activity is balanced by calcium induced release of calcium from the sarcoplasmic reticulum through the ryanodine receptor (CICR). Beginning in the illuminated region the system starts to oscillate and behaves as a pacemaker of the surrounding cells.
We take this procedure as a way to simulate the appearance of extrasystolic beats and the spatially restricted breakdown of the calcium homeostasis under infarctious situations in the working heart.

In Vitro Assay for Lymphocyte Filtration by Filters of the Cardiopulmonary Bypass

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The various parts of cardiopulmonary bypass (CPB: venomidicard, oxygenator, paper filter, fibre) can induce a selective binding and stimulation of leukocytes in a clinical study we found that during surgery with CPB early activated (CD69+) lymphocytes and B cells are selectively lost. In the present study we analysed in *vitro* if this selective loss is due to filtration in the filters of the CPB. In our experiments we used the filters from the venomidicard because in these filters > 50% of the filtered cells were found.
Blood, obtained from healthy adult volunteers, was led five times through the isolated filters. The leukocytes were immunophenotyped before (control) and after filtration (filtered). In addition, cells in the filter were isolated by washing with phosphate buffered saline.
Samples were analysed on a dual-laser flow cytometer. We used four colour antibody combinations:
CD19FITC/CD69PE/CD3PerCP/CD45APC,
CD25FITC/CD54PE/CD3PerCP/CD19APC.
We found a decrease of the fraction of B lymphocytes in the filtered sample from 13% to 8 %. Among the B lymphocytes preferentially the CD69+ fraction was filtered. On the other hand filtration of CD69+ T lymphocytes and NK cells was less efficient. There was no clear selectivity in the filtration of CD25+ T-cells. Our present data are in agreement with the clinical studies and indicate that lymphocytes selectively adhere to the filters of the CPB. Using *in vitro* filtration and subsequent immunophenotyping could be an important tool for quality assessment of biological filters..
(Supported by the Deutsche Stiftung für Herzforschung).

Spontaneous and Induced Apoptosis of Neutrophils: Differences of Normal and G-CSF-Induced Cells

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Neutrophils are short living cells and rapidly undergo apoptosis in vitro. So we sought for a method to delay their early entry into programmed cell death.
Neutrophils were isolated from normal donors and donors treated with G-CSF.
We investigated the apoptosis (CD 95, Fas Ligand, Apo 2.7, Annexin V, MitoTracker™, bcl-2, p53, p21WAF-1), the expression of surface molecules (CD 11b, 16, 54, 62L, 64) by flow cytometry.
Further apoptosis was induced *in vitro* by using an anti-Fas antibody (IgG_3, clone Apo1-3).
Normally there was an increase of apoptotic cells over storage time, best shown with Annexin V staining. We found an expression of bcl-2 in the neutrophils and there were also detectable protein levels of p53. Only in a few patients we could measure an expression of p21WAF-1. G-CSF induced cells constantly expressed lower protein levels of bcl-2 and p53.
Concerning the induction of apoptosis, neutrophils showed a certain order of sensitivity to *in vitro* anti-Fas treatment. (G-CSF *in vivo* < G-CSF *in vitro* < untreated)
Using neoplastic cell lines increasing p53 and decreasing bcl-2 have been described. Using peripheral blood neutrophils, these changes could not be observed. Therefore, a difference between cell lines and primary cells has to be considered and other pathways might be involved in apoptosis of normal, non-malignant myeloid cells.

Spektrale Präzisionsdistanzmessung Mittels Konfokaler Laser-Scanning Mikroskopie in der 3D-Genomanalyse

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Für die Erforschung der dreidimensionalen (3D) Struktur des menschlichen Genoms und deren funktioneller Bedeutung lassen sich in intakten Zellkernen kleine und kleinste DNA-Abschnitte mittels in situ Hybridisierung spezifisch Fluoreszenz markieren. Für die quantitative Analyse der Topologie dieser Marker eignen sich Fernfeld-Lichtmikroskope hoher Auflösung und Präzision. Hier wird die Technik der Spektralen Präzisionsdistanzmikroskopie (SPM) vorgestellt. Das Prinzip beruht darauf, daß die Lokalisation des Baryzentrums ("optischer Intensitätsschwerpunkt") einer Fluoreszenzmarkierung in der Mikroskopie wesentlich genauer als die Auflösung (= Halbwertsbreite der Punktbildfunktion) bestimmt werden kann.
Diskriminiert man Markierungen, deren Abstände kleiner als die Auflösung sind, durch verschiedene spektrale Signaturen, lassen sich diese getrennt lokalisieren und somit ihre Abstände bestimmen. In der konfokalen Laser-Scanning Mikroskopie erhält man so ein "Auflösungsäquivalent" von ca. 50 nm, entsprechend wenigen Nukleosomendurchmessern. Bei dieser Präzision müssen jedoch Abbildungsfehler, d.h. insbesondere der chromatische Versatz mittels Kalibrierpartikel oder in situ durch simultane Mehrfarbenmarkierung eines DNA-Abschnittes kalibriert werden.
Die SPM wurde angewandt, um Distanzen zwischen ausgewählten Klonen der Prader-Willi-Region auf Chromosom 15 zu ermitteln. Zur Verfügung stehen DNA-Proben von vier verschiedenen Klonen, deren genomische Distanz in Kilobasenpaaren bekannt ist. Nach Zweifarben-Fluoreszenz in situ Hybridisierung in menschlichen Fibroblastenzellkernen wurden die Signale mit hoher Präzision lokalisiert und deren Distanzen gemessen. Das Ziel liegt darin, die genomischen Distanzen zwischen den Klonen mit den gemessenen geometrischen Distanzen (in nm) zu vergleichen und somit Erkenntnisse über die Topologie dieser Region zu erhalten.

BCL-2-INDEPENDENT LOCALISATION OF BAX IN LEUKEMIA CELLS BY CONFOCAL LASER MICROSCOPY

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Bc1-2 and bax are members of the bcl-2 family that play a key role in the regulation of apoptosis. Their mechanism of action has been proposed to implicate in vivo homo- and heterodimerizations and an association with intracellular membranes due to their hydrophobic C-terminal regions. For bcl-2, preferential association with mitochondrial membranes has been clearly demonstrated. By contrast, subcellular distribution of bax has been controversially discussed. Thus, bax was suggested to bind mitochondria either as an integral membrane protein or to be targeted to mitochondria by bcl-2. Moreover, recent studies wth bax-transfected fibroblasts and epithelial cells suggested diffuse distribution of bax in living cells which was independent of bcl-2 expression. To investigate subcellular distribution of bax and its relation to bcl-2 we studied leukemia T-cell lines and freshly isolated acute leukemia cells of T-lineage by confocal laser microscopy. To this end, we applied threecolor analysis of cells stained in various combinations for bax, bcl-2, mitochondria, cell nuclei and apoptotic plasma membrane. In living cells, bcl-2 was found to be mainly colocalised with mitochondria. By contrast, we observed considerable amounts of bax which was located out of mitochondria. In almost all samples the distribution pattern was non-diffuse and clearly punctate. Interestingly, these distribution patterns did not depend on intracellular expression levels of bcl-2 and bax measured by flow cytometry in parallel to confocal microscopy. Therefore, our data suggest a discontinuous, punctate subcellular distribution of bax which is to a great extent out of mitochondria and does not depend on bcl-2 localisation and expression.

MIKRODISSEKTIONS-UNTERSTÜTZTE FISH-ANALYSE VON DYSPLASIE UND CARCINOMAIN SITU DER HARNBLASE.

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Ziel: Die gängige Hypothese zur Entstehung des Harnblasenkarzinoms beschreibt getrennte Wege für den papillären Tumor (assoziiert mit Chromosom 9 Aber-ration) und die flachen Neoplasien (assoziiert mit p53 Mutation). Im Rahmen einer Studie zu genetischen Veränderungen früher Stadien des Harnblasenkarzinoms bestand ein wichtiger Teilaspekt in der Untersuchung von multifokalen Carci-nomata in situ (CIS) und mittelgradigen Dysplasien (DII) hinsichtlich Veränderungen von Chromosom 9 und 17.

Material und Methoden: Von insgesamt 19 Patienten wurden 28 CIS und 15 DII untersucht. Von Biopsien, die im Rahmen der endoskopischen Fluoreszenzdiagnostik mit 5-Aminolävulinsäure gewonnen worden waren, wurden 20µm Gefrierschnitte angefertigt, Methylenblau-gefärbt und anschließend das Urothel mikrodisseziiert. Die anschließende Fluoreszenz in-situ Hybridisierug erfolgte an vereinzelten Zellkernen mit biotinylierten Sonden für die Genloci 9q22 (FACC), 9p21 (CDK) und 17p13 (p53). Ausgewertet wurden die Signale von mindestens 60 und höchstens 115 Zellen.

Ergebnisse: Die Verteilung genetischer Veränderungen in den untersuchten CIS betrug p53 (89%) = CDK (89%) > FACC (75%). Synchrone multifokale CIS zeig-ten häufiger gleichartige als divergente Aberationsmuster. Dysplasien zeigten eine insgesamt etwas niedrigere Deletionsrate als CIS mit einer Häufigkeitsverteilung von CDK (73%) > p53 (40%) = FACC (40%).

Schlußfolgerung: Mit diesen Arbeiten wird erstmalig gezeigt, daß CIS schon sehr hohe Deletionsraten von Chromosom 9 zeigen und im Bezug auf dieses Gen nicht von papillären Tumoren getrennt werden können. Die relativ hohe Rate an p53 Mutationen in den flachen Läsionen liegt zahlenmäßig deutlich über dem papillärer Tumoren (Hartmann et al. Verh. Dtsch. Ges. Path. 81:746,1997) und ist schon für Dysplasien gültig.

Mit Unterstützung durch DFG Kn263/7-2 und Mildred Scheel Stiftung: 10-1096-Ha1.

MAPPING THE CELL SURFACE DISTRIBUTION OF ERBB MOLECULES IN BREAST CARCINOMAS.

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ErbB2, a member of the epidermal growth factor (EGF) receptor-type tyrosine kinase receptor family, is overexpressed in breast tumors with poor prognosis. We studied the cell surface association of this receptor with itself and with other cell surface proteins by flow and image cytometric fluorescence resonance energy transfer (FRET) methods using fluorescently labeled monoclonal antibodies. We detected a high degree of homoassociation of erbB2 molecules in unstimulated SK-BR-3 cells. This interaction was enhanced by EGF treatment. In accordance with the EGF-induced redistribution of erbB2, EGF receptor was also in close proximity to erbB2. Image microscopic photobleaching energy transfer measurements detected considerable pixel-by-pixel heterogeneity in the homoassociation state of erbB2. In some regions of cell membranes anomalously high erbB2 homoassociation was observed. We used the shared aperture mode of a scanning near-field optical microscope to visualize erbB2 clustering and to characterize the dimensions of these regions. Our results showed that erbB2 was concentrated in membrane areas with a diameter of 400 nm on unstimulated cells. Activation with EGF, heregulin and monoclonal antibodies increased the diameter of erbB2 clusters to 700 nm irrespective to the activating ligand. In addition to the short-scale association of erbB2 with other erbB proteins (formation of homo- and heterodimers, as detected by FRET), the association of several hundreds of proteins in clusters may have also physiological significance. These clusters may be the initiation sites of transmembrane signaling, the exact mode of activation being determined by other erbB proteins possibly also present in the clusters.

AUTOMATED CLASSIFICATION OF PERIPHERAL BLODD AND BONE MARROW ASPIRATES FROM CHRONIC LYMPHATIC LEUKEMIA (B-CLL) BY THREE COLOUR FLOW CYTOMETRIC IMMUNOPHENOTYPING

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The correct analysis of light chain restriction in B-CLL from flow cytomdric measurements requires thorough investigation because the interpretation of results in case of weakly expressed immunoglobulines may be ambiguous. The aim of this study was to investigate whether CD45/14/20, CD8/4/3, KappalCD19/5, LambdaCD19/5 flow cytometric list mode files were suitable for automated classification by the CLASSIF1 triple matrix algorithm (Cytometry (CCC) 30:275-288(1997), http://vww.biochem.mpg.de/valet/classif1.html).

Bone marrow aspirates (BMA) and peripheral blood leukocytes (PBL) of normal and APAAP/histologically proven kappa and lambda expressing B-CLL patients served as learning set. After completion of the self learning process, BMA were correctly classified in 100.0% of the cases (n=22/26/27) with predictive values of 100.0, 100.0 and 96.3%. Similarly, BMA samples were classified against PBL, as an easier control assay with: 100.0, 96.2 and 96.3% (n=58/26/27) at predictive values of: 100.0, 94.6 and 94.6%. PBL were correctly classified in: 98.3, 91.2 and 100.0% of the cases (n=58/34/38) with predicitive values of: 91.8, 91.2 and 94.1%.

As a first test set, prediagnosed BMA (n=12) and PBL (n=10) samples, unkown to the classifiers, were correctly classified for BMA and PBL in 91.2% (n=11/12) and 100.0% of the cases (n=10/10). In a socond test set, 8 BMA samples and 7 PBL samples, not well classifiable by routine flow cytometric analysis were unambiguously classified by CLASSIF1 analysis.

In addition, CLASSIF1 analysis is significantly faster than the execution of manual or automated measurements, thus fulfilling the requirement for a fully automated on-line analysis of multiparametric data file sets.

Relationship between Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) and Cell Cycle in Lutein Cells

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Introduction

Ovarian follicle cells differentiate to produce pregnancy - maintaining steroids (progestins, i.e., progesterone and related steroids) after ovulation, generating the corpus luteum. The corpus luteum is regressed when fertilization of the released oocytes did not take place. The regulation of this process is not fully understood but may be associated with the expression of transcription factors activating gene products some which are involved in pathways of the cholesterol and lipid metabolism. As peroxisome proliferator-activated receptors (PPAR) may play a role for the differentiation of lutein cells, we were interested in the expression of PPARγ and PPARγ -mediated action on cell cycle progress , a PPAR form which is involved in the adipogenic differentiation.

Methods

The expression of PPARγ in bovine lutein cells (day 12 of the ovarian cycle) was analyzed at the level of the mRNA and ectropic expression by imaging, flow cytometry, and blot analysis. Cell function was tested by progesterone secretion and by the response to the mitogenic drug aurintricarboxylic acid (ATA) and to 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15-d PGJ_2) an endogenous ligand of PPARγ. Cell cycle was analyzed by flowcytometry, using propidium iodide staining after ethanol fixation and RNAse treatment.

Results and Conclusions

The cells (24 h culture) respondeddose-dependently with increasing the progesterone secretion (up to 1.5 fold the basal level) to 15-d PGJ_2. ATA was found to reduce the intracellular PPARγ level and to promote the cell cycle progress, indicating ATA as tool for experimental changes of PPARγ proteins in intact cells and for studying the physiological consequences. The ATA-mediated decrease of PPARγ was accompanied with a reduced progesterone production and a progression of the cell cycle, indicative for a function of PPARγ in both processes. The response to ATA was abrogated by a high dose (> 490 nM) of 15-$dPGJ_2$, suggesting 15-$dPGJ_2$ exerts its effect on steroidogenic activity via PPARγ and a role of the 15-$dPGJ_2$ -PPARγ system for maintenance of a differentiated quiescent stage in lutein cells.

Konzepte und Techniken zur Biophysikalischen Analyse des Zellulären Signalverhaltens von Tumorzellen mit Modularen Mikrosensorarrays zum Möglichen Einsatz in Diagnostik und Therapie

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Tierische und pflanzliche Zellen sind Mikrosysteme mit hohem strukturellen Organisationsgrad und nichtlinearer Signaldynamik [1]. Sie besitzen parallele Datenverarbeitungseigenschaiften [2] und antworten auf physikalische oder chemische Stimuli mit definierten Signalen [3,4].

Im Rahmen systemanalytischer und experimenteller Untersuchungen zum Wachstumverhalten von lumorzeilen und deren möglicher chemotherapeutischer beeinflussung haben wir ein Meßsystem entwickelt, das an Zell- und Gewebematerial diese zelluläre Signale mit geigneten Mikrosensoren on-line und real time registrieren kann [5,6]. Dieses System wird zur Zeit in einem Verbundprojekt unter Förderung des BMBF mit verschiedenen Partnern realisiert und gestestet. Zellen aus Zellkultur und Biopsien können konventionell vorkultiviert und für die Dauer der Messung in eine mit verschiedenen Sensoren bestückte Kammer eingesetzt werden. Mikrosensoren für pH, Sauerstoff, Temperatur, Ionen und elektrische Wechselwirkungen der Zellen stehen entweder in unmittelbarem Kontakt zu den Zellen oder sind im Strömungskanal des Nährmediums positioniert [7,8,9]. Zusätzlich ist das Zell- oder Gewebematerial mikroskopierbar, so daß alle Standardtechniken zu Visualisierung intrazellulärer Signalverarbeitungsprozesse eingesetzt werden können. Anhand verschiedener Experimente zur chemotherapeutischen Beeinflussung von Tumorzellen [10,11] werden die verschiedenen möglichen Versionen des Systems sowie Anwendungen in der biomedizinischen Forschung präsentiert und zur Diskussion gestellt .

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POSTER

ABSTRACTS

DETERMINATION OF DNA-PLOIDY IN TRANSITIONAL CELL CARCINOMA OF THE BLADDER IN CORRELATION TO THE HISTOPATHOLOGICAL STAGE AND CLINICAL COURSE

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Background: According to Bergkvist et al and Ooms et al, the stage and histopathological grade of a tumor are not sufficient to judge its biological aggressiveness in respect to recurrence, invasion and metastatic growth. Since histological grading is subjective and not uniform, it is necessary to assess the grade of malignancy and tumor stage objectively by means of prognostic methods.

Methods: In 147 patients with urothelial carcinoma of the bladder, the ploidy, deoxyribonucleic acid (DNA) heterogeneity and cell cycle count phases in the tumor were analyzed by single cell DNA cytophotometry in order to determine new prognostic factors in addition to those already known (stage and grade). Patients were studied for 1 to 12 years, mean 6,7 years.

Results: The spread of bladder carcinoma was determined in accordance with the classification of the International Union Against Cancer. A total of 76 patients (52 %) had stage pTa to pT1, 35 (24 %) stage pT2, 25 (17 %) stage pT3 and 11 (7 %) stage pT4 disease. The tumors were classified histologically according to the guidelines issued by the World Health Organization. Histological grade 2 (53 %) was most prevalent. Grade 3 tumors were present in 24 % and grade 1 tumors in 23 % of the patients.

There was a correlation between tumor stage and DNA ploidy. The cell lines were aneuploid in 38 % of the patients with stage pT1, 64 % with stage pT2 and 88 % with stage pT3 tumors.

Radical cystectomy with pelvic lymphadenectomy was performed in 32 patients with stage pT2 (pT1 to pT2) and 9 with stage pT3 disease. Of the 21 stage pT2 and 9 stage pT3 cancer patients (that are 66 % of patients with cystectomy), 2 to 4 had positive lymph nodes. Of the cystectomy specimens obtained intraoperatively from patients with stages pT2 to pT3 disease, 45 % showed secondary microscopic invasive tumor foci. All of these tumors were aneuploid without exception and showed multiple aneuploid cell lines.

A significant correlation was found between DNA cytophotometry and the clinical course of the disease. Patients with diploid tumor cell lines (2c region) had no metastases and no local tumor progression for up to 10 years. Patients with multiple aneuploid tumor cell lines (3c, 5c, 7c and higher) suffered recurrence and local tumor progression within 4 to 16 months, mean 7 months. The patients died of the tumor 26 months after primary diagnosis. The difference in tumor recurrence and in tumor progression between patients with aneuploid and diploid tumors was significant ($p > 0.001$).

Conclusion: In our study, 43 % of the patients had muscle-infiltrative disease at cystectomy. All of this tumors were analyzed without exception and showed multiple aneuploid cell lines. This finding clearly indicates that many of these cancers may express their potential for infiltration and metastasis early enough in the clinical course so that cancer-specific mortality will occur, cystectomy having presumably been done in a timely fashion. The results obtained in the multi-variance analysis, showed that only tumours with pT stage (P=0,0083) and DNA Histogram (P=0,0041) had a decisive effect on the prognosis of the patient.

Carboxyfluorescein Labelling and Efflux as Inidicators for Acidification by Lactic Acid Bacteria

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The viability and vitality of lactic acid bacteria are essential in dairy fermentations, such as yogurt and cheese making. The traditional techniques for determination of viability and vitality are plate counts and acidification tests. These experiments require long incubation times and give limited information. Fluorescent techniques however provide the possibility for fast measurements with high sensitivity and the potential of single-cell analysis. Fluorescent probes can be used to measure various physiological parameters, such as enzyme activities, membrane integrity and cytoplasmatic pH, by fluorescence microscopy, spectrofluorimetry, and flow cytometry. Fluorescein and its derivatives are used extensively for viability measurements and intracellular pH measurements of bacteria, yeasts and mammalian cells. We tested the use of carboxyfluorescein (cF) to indicate the viability and vitality of lactic acid bacteria and to study stress responses.
Lactococcus lactis ssp lactis ML3 could readily be labeled with cF by incubation with the non-fluorescent precursor carboxyfluorescein diacetate (cFDA). The labeling capacity was lost after heat treatment at 70 °C. In mixtures of nontreated and heat-killed cell suspensions the two subpopulations could be discriminated by simultaneous lightand fluorescence microscopy. Non-energized cells retained the cF well, whereas energizing with lactose caused an immediate and rapid transport out of the cell. We compared fluorescent parameters with plate counts and acidification capacity after subjection to heat stress, freezing and low pH. From these experiments we concluded that a combination of cF-labeling and efflux gave a good indication for the acidification capacity. Advantages of this fluorescent method compared to the traditional methods are the required time (about one hour) and the potential of analyzing subpopulations. Further studies using different lactic acid bacteria strains and flow cytometric analysis are in progress.

Flow Cytometric Detection of Apoptotic Fractions During Cell Cycle in Human Mammary Carcinomas Using TUNEL-Method

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Apoptosis has since been defined in general terms as programmed cell death or cell suicide. The most reliable method of identification of apoptotic cells is via analysis of their morphology by microscopy. DNA-cytometry shows apoptotic cells in a hypodiploid peak as a sign of loss in DNA, but it is not possible to position them with respect to their cell cycle phase. We correlate single parametric DNA-cytometry with multiparametric cytometry.

The TUNEL-method was examined for monitoring spontaneous apoptotic response in human mammary carcinomas. We analysed our routine material by multiparametric flow cytometry, based on simultaneous analysis of cellular DNA-content and the detection of DNA-strand breaks. The strand breaks labeling with fluorescein dUTP by terminal deoxynucleotidyl transferase has the advantage of making it possible to estimate the cell cycle distribution of both the apoptotic and uneffected cell population.

The percentage of spontaneous apoptotic cells in our probes ranged from 1,5 - 25%. TUNEL positive cells are fast higher than in detected sub-G0/1-peaks in DNA-cytometry. Diploid cell population in aneuploid tumors shows no or only weak apoptotic cell population, while aneuploid tumor cell populations mainly apoptosis respective strand breaks observed in late S-phase fraction.

Flow-Cytometrische Untersuchung Bakterieller Populationen zur Situationserkennung bei Nichtstationären Kontinuierlichen Prozessen

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Poly-ß-hydroxybuttersäure (PHB) wird von einer Vielzahl von Bakterien im Ergebnis von Imbalanzen im Nährstoffangebot intrazellulär synthetisiert. Werden Zellen von *Methylobacterium rhodesianum* MB126 auf Methanol wachstumslimitierenden Bedingungen, wie zum Beispiel Stickstoff-Limitation, ausgesetzt, synthetisieren sie PHB, nachdem sie einen Zellzustand mit doppelten DNA-Gehalt erreicht haben.
Es gilt nun, die Synthese der ökologisch äußerst günstigen PHB auch ökonomisch interessant zu gestalten. Dazu ist es notwendig, eine optimale Prozeßführung zu erarbeiten, welche die unterschiedlichen Leistungen des Bakteriums *Methylobacterium rhodesianum* MB126 in Abhängigkeit des vorherrschenden Nährstoffangebotes berücksichtigt. Es wird dabei von der Hypothese ausgegangen, daß diese differierenden Leistungen an verschiedene, metabolische Zustände der Bakterienzellen gekoppelt sind.
Die Grundlage der hier vorgestellten Kontrollstrategie bildet deshalb eine strukturierte, segregierte Systemphilosophie. Dabei werden Informationen über die Zustandsverteilung bakterieller Kulturen mit Hilfe der Methode der Flow-Cytometrie gewonnen. Auf der Basis einer gleichzeitigen Charakterisierung von Übergangszuständen mittels Standardmethoden (integrale Untersuchungen) sowie durch die mehrparametrische Methode der Flow-Cytometrie (lokale Untersuchungen) können gemessene Zustandsverteilungen typischen Zuständen des biotechnischen Prozesses zugeordnet werden.
Die somit gewonnenen Informationen werden zur Bildung und Parameterisierung eines einfachen Prozeßmodells genutzt. Dieses strukturierte, segregierte Modell unterscheidet einerseits, bezogen auf eine Bakterienzelle, zwischen metabolisch aktivem und inaktivem Anteil und berücksichtigt andererseits, betrachtet man die gesamte Bakterienkultur, zwei Zellzustände. Dabei ist der metabolisch aktive Zellanteil entweder in der Wachstums- oder der Synthesephase. Den inaktiven Zellanteil stellt die in Form von Granula akkumulierte bzw. deponierte PHB dar.
Ein Modul Erweitertes Kalman Filter wird präsentiert, in dessen rekursiven Filteralgorithmus zur Schätzung der Zustände nichtlinearer Systeme dieses Modell integriert ist.
Auf der Grundlage dieser Situationserkennung, soll nach einer initialen Synchronisation, zur Optimierung der Zustandsverteilung, durch periodische Veränderungen in der Zusammensetzung der zufließenden Nährlösung eine zeitliche Trennung von Wachstumszuständen und Zuständen des "overflow metabolism" in der Kultur erreicht werden.

Activated Neutrophils and Lymphocytes: Change of Cell Surface Molecules during 72 Hours of Storage in Three Different Anticoagulants

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Alteration of cell surface molecules of peripheral blood neutrophils and lymphocytes, reflecting altered activity, is of clinical interest. A dysfunction of these two cell types is associated with several diseases, e. g. CR3 deficiency, chronic granulomatous disease, neonatal sepsis or the adult respiratory distress syndrome (ARDS) of the neutrophil-system and concerning the lymphocytes, graft versus host disease or diminished defense mechanisms against viral and intracellular infections has to be mentioned.
The aim of our study was to determine the influence of three different anticoagulants: lithium-heparin (HEP), ethylenediamine-tetraacetic acid (EDTA) and citric acid (ACD) and the duration of storage on the expression pattern of cell surface molecules.
In our study blood from healthy donors was preincubated with lipopolysaccharide (LPS) (1µg/ml) or IFN-gamma (1000 U/ml) and stored at room temperature.
After storage time of 0, 24, 48, 72 hours cells were analysed by flow cytometry. We utilized a time saving whole blood analysis, FITC- and PE-labeled monoclonal antibodies for detecting the following cell surface molecules: neutrophils: CD 11a, 11b, 14, 16, 18, 32, 54, 62L and 64; lymphocytes: CD 3, 4, 8, 19 and 25.
The obtained means of fluorescence intensity showed no difference between HEP and ACD in contrast to cells which were stored in EDTA. These results bear resemblance to those obtained in an earlier study with non-activated granulocytes. Lymphocyte surface markers did not differ to a significant extend or decreased within 72 hours of incubation, indiscriminately which anticoagulant was used.
We consequently recommend to store whole blood samples, assumed to be activated in vivo either by LPS or cytokines, under room temperature conditions in HEP or ACD and the incubation time should not exceed 48 hours.

CORRELATION OF FCM WITH FAB SUBTYPES IN ACUTE MYELOID LEUKEMIA (AML)

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FCM (flow cytometry) is increasingly used in diagnostics and classification of hematopoietic malignancies. In this study we tried to explore the clinical and diagnostic value of immunophenotyping AML using the flow cytometry method to a variety of leukocyte differentiation antigens.
Thirty-three adult patients with clinical and cytological diagnosis of AML were analysed in this study. Median age was 59 years, (range 27-49), 16 patients were females. In most cases (30) the materials that were analysed were both peripheral blood and bone rnarrow samples (91%), in two cases only bone marrow aspirate was analysed (6%) whereas just in one case only peripheral blood is used for analysis (3%).
The population of blast cells was analysed with specific monoclonal antibodies directed at myeloid (CD13, CD14, CD15, CD33, CD11b), lymphoid (CD19, CD7, CD10) and stem cell (CD34, MPO, HLA D/DR) antigens.[1 2] Results were classified according to both Uthman (1997) and Jennings (1997) proposal, based on the positive/negative surface antigen expression of the blast cells, and correlated to clinical and routine cytological FAB classification.[3]
FCM results obtained from peripheral blood and from the bone marrow samples were fully comparable both in mean percentage of blast (67.8% blast cells in peripheral blood samples relate to 66.0% in bone marrow aspirate) and in FCM classiflcation.

The main diagnostic significance of the myeloid antigens on peripheral blood samples and bone marrow aspirates have CD13 and CD33 according to the number of positive cases analysed. Other antigens (CD11b, CD14, MPO) have shown less positivity. In majority of analysed samples there is bright positivity of CD7 on the cells that already are CD33 positive (so called "double positivity CD7+ CD33+").

Based on the positive/negative surface antigen expression for every patient sample analysed the belonging to certain FAB subtype is confirrned in 27 cases (27/33;82%). Detailed clinical characteristics and course of the disease is presented for non-confirmed cases. Although the confirmation rate is high, those results indicate that FCM classification methods may not be fully explored and further studies and improvements are warranted.

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QUANTITATIVE BESTIMMUNG DES EGF-REZEPTORS BEIM ENDOMETRIUMKARZINOM UND NORMALEN ENDOMETRIUM DURCH MULTIPARAMETRISCHE DURCHFLUßZYTOMETRIE

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Das Thema der Überexpression und Bedeutung des EGF-Rezeptors beim Endometriumkarzinom wird kontrovers diskutiert. Insbesondere technische Schwierigkeiten bei der Identifizierung und Quantifizierung des Rezeptors in Gewebshomogenaten durch Rezeptorbindungsassays und die Immunhistochemie sind hierfür ursächlich zu diskutieren.
In unserer Arbeit wurde die EGFR-Expression beim Endometriumkarzinom (n=47) und normalen Endometrium (n=6) quantitativ durch eine multiparametrische durchflußzytometrische Analyse bestimmt. Hierzu wurde ein monoklonaler EGF-Antikörper (Maus, EGF-AB-1, Fa. Dianova) und ein FITC-konjugierter Ziege-Antimaus-Antikörper (Fa. Becton Dickinson) eingesetzt. Die Quantifizierung der gebundenen Antikörper erfolgte über den simultanen Einsatz von Microbeads (Fa. Quantum Simply Cellular) mit definierter Anzahl an antigenen Bindungsstellen. Die Tumorzellen wurden durch eine simultane DNAFärbung (Propidiumjodid) und die normalen endometrialen Zellen durch eine Zytokeratinfärbung identifiziert.
Beim Endometriumkarzinom ließen sich 2,31 x 10^3 (Median, min. 0,00 bis max. 22,26 x 10^3) Bindungsstellen pro Zelle und beim normalen Endometrium 6,83 x 10^3 (Median, min. 4,94 bis max. 11,57 x 10^3) Bindungsstellen pro Zelle nachweisen. Insgesamt zeigten lediglich 2 Endometriumkarzinome (4%) eine Überexpression des EGFRezeptors im Vergleich zum normalen Endometrium. Bei 81% konnten wir eine geringere und bei 15% eine innerhalb der Grenzen des Normalgewebes liegende Expression des Rezeptors nachweisen.
Es existieren Hinweise in der Literatur, daß Karzinome mit einer Überexpression des EGF-Rezeptors eine kleine Untergruppe mit schlechter Prognose für die Patientin darstellt. Unsere innerhalb dieser prospektiv angelegten Studie erhobenen Daten weisen darauf hin, daß lediglich bei einem kleinen Teil der Endometriumkarzinome eine Uberexpression vorliegt und daß der Rezeptor in der Mehrzahl der Fälle downreguliert ist. Ein ähnliches Verteilungsmuster konnten wir bereits für das Zervixkarzinom zeigen. Ob sich die vermutete klinische Relevanz in unserem Patientenkollektiv bestätigen läßt, wird derzeit durch weitergehende Follow-up-Erhebungen untersucht.

Zur Qualitätskontrolle der Leukozytenmessung im menschlichen Blut: Circadiane Veränderungen sind größer als die Impräzision der verwendeten Messverfahren

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Vorhaben: Um bei der Messung der Leukozyten und ihrer Subpopulationen im Blut präanalytische Einflüsse untersuchen zu können, werden circadiane Abweichungen bei Blutleukozyten bestimmt und mit der Impräzision ihrer Messverfahren verglichen.
Material und Verfahren: Venöses EDTA-Blut wurde von gesunden Männern im 4-h-Intervall über 48 h gewonnen und die Leukozytenpopulationen mittels monoklonaler Antikörperreagenzien nach Lyse im FACScan (Becton Dickinson) bestimmt [1]. Die Leukozyten- und Erythrozytenzahl wurde im Cell-Dyn 1600 (Sequoia Turner) gemessen. Statistische Auswertung erfolgte nach Nelson et al. [vgl.1].
Ergebnisse: Es wurden folgende Abweichungen (0. bis 99. Perzentil als Prozent des Medians in M/L) im Vergleich zur Amplitude in % des Mesors bzw. zum Variationskoeffizienten VK der intra- bzw. interseriellen Impräzision sowie die Peak Time mittels Cosinorrhythmometrie berechnet für Leukozyten: 61%-121%, Amplitude: <8%; VK: <4%, Peak: 21.00 h; neutrophile Granulozyten: 66%-147%, Amplitude: <7%, VK: <4%, Peak: 19.00 h; eosinophile Granulozyten: 45-146%, Amplitude: 25%, VK: <7%, Peak: 07.00 h; Lymphozyten: 79%-127%, Amplitude: <11%, VK: <6%, Peak: 23.00 h; Monozyten: 50%-140%, Amplitude: 13%, VK: <8%, Peak: 20.00 h; CD3+ T Zellen: 73%-144%, Amplitude: 14%; VK: <5%, Peak: 23.00 h; CD8+ T Zellen: 75%-142%, Amplitude: 20%, VK: <10%, Peak: 24.00 h; CD4+ T Zellen: 70%-147%, Amplitude: 12%, VK: <8%, Peak: 23.00 h; CD19+ B Zellen: 68%-147%, Amplitude: 28% VK: <13%, Peak time: 21.00 h; NK Zellen (CD16+56+3-): 32%-163%, Amplitude: 31%, VK: <16%, Peak: 11.00 h.
Folgerung: Es werden signifikante circadiane Veränderungen von Leukozytenpopulationen im Blut gefunden, deren Abweichungen vom Median bzw. der Amplitude bei Cosinorrhythmometrie [vgl.1] größer ist als der VK der Impräzision ihrer Bestimmungsverfahren. Da auch die Peak Time variiert, muß bei der Analyse von Leukozyten und ihren Populationen die Abnahmezeit der Blutprobe zur gleichen Tageszeit erfolgen, um präanalytische Fehler, z.B. Abweichungen von Normalbereichen, zu vermeiden.

[1] T.O. Kleine et al. J Interdisc Cycle Res 1993;24:236-9.

Zur Qualitätskontrolle der Liquorzell - Analyse: Modifikationen der Präparationstechnik führen zu unterschiedlichen Zellbildern

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Die Zellanalytik im Liquor cerebrospinalis erfordert bei geringen Leukozytenzahlen (Normalbereich: 0-5 M/l für Lumballiquor) eine Zellanreicherung, um statistisch repräsentative Zellzahlen (≥100 Leukozyten) untersuchen zu können. Diese Technik soll a) einfach, schnell, schonend und reproduzierbar sein; b} alle Zellen (Leukozyten und Erythrozyten) erfassen (Zellausbeute ≥50%), ohne daß die Zellen während der Aufarbeitung denaturieren; c) bei geringem Probenvolumen möglichst mehrere Zellpräparate liefern sowie zellfreien Überstand für weitere Analysen; d) die Zelldarstellung soll der des konventionellen Blutausstrichs vergleichbar sein. Es sind mehr als 10 Präparationstechniken beschrieben worden, aber keine erfüllt alle oben geforderten Bedingungen. Hier werden 6 gebräuchliche Techniken vergleichend quantitativ (Angabe von Mittelwerten) und qualitativ nach den Kriterien a-d untersucht.
Ergebnisse:
I (a,b,c) :Phasenkontrastmikroskop-Technik (Sörnas 1967):
Zellausbeute: >60%. Normalbereiche im Lumballiquor: Lymphozyten: 87%, monozytäre Zellen: 11%, Makrophagen: 2% (Referenzmethode).
II (a,b,c): Objektträgerverfahren (Kleine et al. 1977): Vitalfärbung nativer Liquorzellen in 20 µl Probe: Zellausbeute: >50%; Anteil nicht differenzierbare Zellen: '10%.
III (a,d): Zytozentrifuge Shandon Cytospin I mit <400 µl Probenvolumen und Anfärbung des Zellpellets nach Pappenheim (Kleine 1980): Zellausbeute: <10%; Anteil nicht differenzierbarer Zellen: <20%.
IV (a,d): Sedimentkammer Sayk I (Sayk 1960; Modifikationen: Lehmitz 1991; Lehmitz & Kleine 1994): Zellausbeute auf PDDA-beschichteten Objektträgern: <10%; Anteil nicht differenzierbarer Zellen: <30%.
V (a,b,d): Sorptionskammer (Lehmitz & Sayk 1991): Zellausbeute auf PDDA-beschichteten Objektträgern: <50%.
VI (a,b,c,d): Hettich Zytozentrifugentechnik mit Vorzentrifugation (Lehmitz et al. 1994; Lehmitz & Kleine 1994): Zellausbeute auf PDDA-beschichteten Objektträgern: <60%.
Folgerung: Von den 6 Präparationstechniken scheiden I und V für die tägliche Routine aus Praktikabilitätsgründen aus. III und IV erfüllen nicht die oben geforderten Kriterien, teilweise 11. Die Hettich Zytozentrifugentechnik mit Vorzentrifugation erfüllt weitgehend die oben geforderten Kriterien einer optimalen Präparationstechnik mit einer Zelldarstellung, die von I, II, IV und V deutlich abweicht.

Entwicklung und Anwendung elektronischer Schnitte und eines virtuelles Mikroskopes für die Auswertung von zytologischen und histologischen Präparaten

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Hintergrund: Die Arbeit mit dem Mikroskop ist noch heute eine sehr ermüdende Tätigkeit. Die zur Zeit erreichbaren Bildanalyse-Systeme können für die Sicherung spezifischer Bilder angewendet werden, jedoch die Einstellung des Mikroskopes, Fokussierung wird manuell gemacht.
Ziele: Erstellung eines elektronischen Schnittes, wo alle biologisch wichtigen Bildsegmente eines Schnittes digitalisiert gesichert werden. Evaluierung der Anwendung eines komputer-basierten, virtuellen Mikroskopes für die Analyse der elektronischen Schnitte aus zytologischen und histologischen Präparaten auf einem Bildschirm eines Komputers, ohne die Anwendung eines Mikroskopes.
Methoden: Die X-Y-Z Bewegung eines Scanningtisches, die Objektivwechsel, die Filterwechsel eines Zeiss Axioplan 2 MOT Mikroskops wurde durch das RS232 Interface von einem Pentium II, 300 Mhz PC kontolliert. Die angewendete Kamera war das Grundig FAC 830, durch RS 285 kontrollierte Kamera. Es wurde eine Screen Machine II Karte für die Bilddigitalisierung angewendet. Die Schnitte wurden mit 400x - Vergrösserung digitalisiert. Die Auflösung des Systems war 0.165 μm^2 / pixel. Die Bilder wurden in JPEG Format gesichert. Bei einer Biopsie von der Grösse 8x12 mm, wurden insge-samt 3000 Bilder aufgenommen. Für die Simulation der mikros-kopischen Funktionen (Bewegung der Bildsegmente, Veränderung der Vergrösserung, Fokussierung) wurde ein Programm unter Borlands C++ Builder für das Windows NT Operationssystem entwickelt. 10 H/E gefärbte klassische, histologische Präparate und 10 H/E gefärbte zytozentrifugierte Mamma Biopsien, wurden mit dem optischen und virtuellen Mikroskop ausgewertet.
Ergebnisse: Die Bilder einer Biopsie oder eines Zytozentrifugen - Präparates benötigen weniger als 200 MB Harddisk - Kapazität. Die verschiedenen Bildsegmente können mit einer Genauigkeit von 0.5 µm zueinander angepasst werden.Die Bewegung der Bildsegmente ist in diskreter oder kontinuerlicher Form machbar. Die Vergrösserung ist zwischen 0x und 400x kontinuierlich zu verändern. Eine pseudoelektronische Vergrösserung zwischen 400x und 600 x gibt noch immer ein qualitativ akzeptables Bild. Die Diagnosen mit dem reellen und virtuellen Mikroskop ergaben dieselbe Diagnose.
Konklusion: Durch die erreichbare Qualität der elektronischen Schnitte und die Funktionalität des virtuellen Mikroskopes ergibt sich eine neue digitale Alternative gegenüber der klassischen Mikroskopie.

Anwendungsmöglichkeiten der Durchflußzytometrie bei der Antibiotikaresistenzbestimmung

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Aufgrund der schnellen Erfassung von Streulicht- und Fluoreszenz-lichteigenschaften von Zellen eignet sich die Durchflußzytometrie zur raschen Bestimmung von Antibiotikaresistenzen bei Bakterien.

Der Einsatz von membranpotentialsensitiven Fluoreszenzfarbstoffen wie $DiBAC_4(3)$ und $DiOC_5(3)$ ermöglicht eine gute Unterscheidung intakter und geschädigter Bakterienzellen. Unabhängig vom Wirkungsmechanismus der Antibiotika ist der Zusammenbruch des Membranpotentials ein sicherer Indikator für eine Schädigung der Bakterienzellen. Eingesetzt wurden die Penicilline Mezlocillin, Piperacillin, Penicillin G und Oxacillin, die Cephalosporine Cefazolin und Cefuroxim, das Fluochinolon Ciprofloxacin und das Aminoglykosid Gentamicin.

Für die häufigsten humanpathogenen Keime wurde die Sensitivität für verschiedene Antibiotika bestimmt. Die eingesetzten gramnegativen Keime waren Escherichia coli, Klebsiella spp., Pseudomonas spp., Citrobacter spp., Acinetobacter spp. und Proteus spp. Als Vertreter grampositiver Keime wurden Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus ssp., Enterococcus faecium und Enterococcus faecalis untersucht.

Nach Anzucht der Bakterien bis zur logarithmischen Wachstumsphase wurden relevante Antibiotika und der Farbstoff zugegeben. Bereits nach 90 - 120 Minuten Inkubationszeit konnten zuverlässige und mit etablierten Routineverfahren (NCCLS) übereinstimmende Aussagen über die Antibiotikaempfindlichkeit der Keime gemacht werden. Diese Verkürzung der Analysezeit im Vergleich zu anderen Verfahren der Antibiotikaempfindlichkeitsbestimmung kann durch eine durchflußzytometrische Analyse aus Direktmaterial wie Liquor oder direkt aus Blutkulturen noch erheblich gesteigert werden.

Isolation and Phenotypic Characterization of Rat Intestinal Intraepithelial Lymphocytes and Investigations for There Role in Chronic Rejection of Intestinal Allografts

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Introduction: Intestinal intraepithelial lymphocytes (IEL), located within villus epithelium, are the first line of lymphoid cells exposed to orally absorbed foreign antigens. Presently, little is known about the possible influence of chronic rejection (CR) through IEL, one of the main complications after small bowel transplantation (SBT). We modified a method described by Kearsey and Stadnyk (J. Immunol Meth 194: 35, 1996) for isolation of IEL with high purity. With this procedure we investigated the composition of IEL after SBT and during CR.

Methods: One-step orthotopic SBT was performed in allogeneic (BN-LEW) and syngeneic (LEW-LEW) rat strain combinations. At different time points IEL were isolated from epithelium layer by vibrating on a vortex mixer and were purified by Percoll centrifugation.. Subsequently three color flow cytometic analysis was performed. Cell samples were analyzed using a FACScan and data were acquired using LYSIS II software.

Results: The rat IEL population - 1-3x10^6 IEL/rat were reproducibly isolated - consisted of 96-98% leukocyte common antigen-positive cells. Within this population, CD8+ T cells were predominant (70-80%) and most of them expressed the aß T cell receptor (up to 75%). Furthermore, IEL contained a sizable population of γδ CD8+ T cells up to 25%. A small population (5-15%) coexpressed CD4 and CD8 molecules. B cells were found at very low levels (< 5%), indicating low contamination with cells from the lamina propria that lies undemeath the epithelium and contains 60% B cells. Normally, the amount of NK cells within the IEL was under 10%. In contrast, after allogeneic SBT they increased and reached a maximum of nearly 50% in the phase of CR. In addition γδ CD8+T cells increased up to 45%.

Conclusions: This procedure allows isolation of highly purified IEL for phenotypic flow cytometry analysis. Differential investigations of IEL after intestinal transplantation are now possible with this method. We have shown that NK and f T cells changed dynamically after allogeneic SBT and reached a maximum in the period of chronic intestinal rejection. These data appear to indicate an important role of these cells in the regulation of chronic rejection.

Modelling the Synchronisation Phenomena of Photolytically Induced Focal Calcium Oscilations in Reconstructed Cardiac Tissue

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Following to the enzymatic digestion of ventricular tissue and seeding of the single cells to cell culture dishes the solitary cells close contact one to another and reconstitute a two dimensional contracting tissue. The outspread of excitation from a focal point in the tissue can be measured using calcium imaging methods under video frequency. Here the onset and growth of oscillating regions can be visualized.

We use the resulting data sets to specify the parameters of a coupled differential equation of the reaction-diffusion type. Besides the formulation of the diffusion by the Fick's laws the equation includes terms for the cytosolic processes of calcium release into and sequestration of calcium out of the cytosol.

$$\frac{\partial c}{\partial t} = D(c(x,t))\frac{\partial^2 c}{\partial x^2} + \frac{\partial D}{\partial x} * \frac{\partial c}{\partial x} + (1 - R(c,x,t)) * E(c(x,t)) - A(c(x,t))$$

Herein the activity of the sequestration process is directly dependent on the cytosolic calcium concentration and has no refractory properties, while the term representing the calcium liberation has a combined calcium and time dependent refratory state. This way the cytosolic calcium diffusion and the coupled activities of the sodium/calcium-exchanger and the Ryanodine receptor can be numerically simulated.

The measurements of the calcium dynamics in heart cells show phenomena such as the onset of a cytosolic calcium oscillator, the synchronization of independent local oscillators and the nonlinear propagation of excitation. These phenomena can be simulated with the coupled differential equation.

Localization of Small Labelled Sites using Spatially Modulated Excitation Microscopy

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For examinations of specific chromatin regions in chromosomes and interphase nuclei, labelling of DNA targets using fluorescence in situ hybridization and the application of fluorescence microscopy are state of the art techniques. For the determination of the functional topology of the genome, high accuracy distance measurements of small labelling sites are required.
To obtain a higher localization accuracy of small targets, interferometric illumination has been introduced. Using a beamsplitter, two beams of light appropriately coherent were superposed in the object area of the microscope resulting in a standing wave field. A variation of the cross angle between the beams leads to a variation of its spatial frequency. By shifting the optical path length of one of the excitation beams, the standing wave field is shifted in the sample. To obtain unequivocal information about object loci, the sample must be thin enough in relation to the wavelength of the fringes. The examined fluorescence targets have to be smaller than half of the wavelength of the standing wave field. New approaches use spatial modulated illuminations in different ways (spatially modulated excitation fluorescence microscopy). For increasing the resolution, multiple beams in axial and/or lateral directions can be applied. Several setups for different demands have been built. For high resolution measurements thermally invariant mounting devices have been developed. To overcome the information ambiguity caused by the standing wave field technique described, stage controlled optical sectioning through the object was applied. For each fluorescent target, a point spread function (PSF) was obtained representing the excitation modulation enveloped by the shape of the epifluorescence PSF. From these data the mean maximum (optical fluorescence bary centre) was determined by an appropriate analysis algorithm. Taking into account a series of the equidistant maxima, the error of localization is in the range of a few nanometers even for small fluorescent targets.
In the case of very short distances (below the resolution limit of the epifluorescent PSF) targets of different spectral signatures were used. By means of an additional excitation wavelength and multi bandpass filters a reliable distance measurement between closely connected spots can be provided (spectral precision distance measurement).

Adaptation of HL-60 to Oxidative Stress by Hydrogen Peroxide

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Problems in environmental protection, questions in the pathogenesis of a lot of diseases and an increasing interest in elucidation of unspecific resistance mechanisms and last but not least the broadened possibilities in methods led in the last years to a growing attention for production of and protection against reactive oxygen species. Beside microorganisms as targets of resistance mechanisms it is also interesting to know how do mammalian cells protect themselve. One possibility could be the adaptation of these cells which was described only for fibroblasts (Spitz, D.R. et al. J.Cell.Physiol. 131 (1987) 364; Wiese, A.G. et al. Arch.Biochem. Biophys. 318 (1995) 231). In HL-60 and using hydrogen peroxide as the source for reactive oxygen radicals we investigated the influence of pretreatment of these cells with different doses of H_2O_2. We wanted to know whether non or mildly toxic doses result in a state of higher resistance and if this effect could be influenced by drug induced cell differentiation.

The pretreatment was done with 25 to 75 µM hydrogen peroxide (differentiated ceils: 75-300 µM) at 10^9/l and 37°C for 90 minutes followed by washing and further normal culture for 22 hours. The challenge was performed at a range between 75 to 1200 µM hydrogen peroxide (differentiated cells: 300-2400 µM) for 90 minutes. The impairment of cells was measured by the FDA-PI-test (30 nM fluorescein diacetate, 18,7 µM propidium iodide, 120 min., room temperature). By acquisition of green and red fluorescences by flow cytometry the test allows the discrimination between vital, apoptotic and necrotic cells. The degree of differentiation induced by DMSO,1,25-dihydroxyvitamin D_3 and Na-butyrate was assessed by flow cytometric phenotyping (CD11 b, CD14, CD16, CD45) and the resistance against H_2O_2 compared with blood monocytes and neutrophils.

The pretreatment of undifferentiated HL-60 led to a significant increase of the adaptation index (ratio of the percentages of living and pretreated cells to living but not pretreated cells) after challenge. We found indices up to 1,6, that means the survival rate goes up to 160 %. In DMSO-differentiated cells we found no adaptation effect (adaptation index 1,0).

Based on our results we conclude that this kind of adaptation depends on cell differentiation.

THE CELL DEATH BY HYDROGEN PEROXIDE - DETECTION BY FDA-PI-TEST

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Depending on the system and the kind of its inducer apoptosis is more or less accompanied by the classical cell death, the necrosis. Hydrogen peroxide belongs as a toxic chemical to the group of noxes whose application results often in both kinds of death. In many of these investigations the rate of apoptosis was determined as exact as possible without watching the fate of the other cells. This was our reason to proof and adapt the well known viability test using Propidium Iodide (PI) and Fluorescein Diacetate (FDA) for the simultaneous and quantitative determination of viability, apoptosis and necrosis by flow cytometry.

Indicator of viability is the retained fluorescein after cleavage of FDA by cellular esterases. Apoptotic cells can not retain fluorescein in the same degree (Ormerod, M.G. et al., Cytometry 14 (1993) 595). This change appears early, remains stable for a long time while apoptosis is running down (Frey, T., Cytometry 28 (1997) 253) and shows good correlation with Sub-G1-detection. Using Calcein AM, like FDA an acetoxymethylester derivative for cell loading but more resistant against leaking, we could show, that the activity of esterases in an apoptotic cell is not substantially impaired. The myelopoietic cell line HL-60 driven into an apoptotic state shows significant reduction in its ability to retain fluorescein. Under optimal staining conditions (30 nM FDA, 18,7 µM PI, 120 to 180 min, room temperature) and using H_2O_2 in a range of 10,0 µM to 2,5 mM we found a decrease in fluorescence intensity by 85 % permitting a clear discrimination between living and apoptotic cells. The rate of necrotic cells spans between 6,0 % and the considerable value of 67 %. Although this impairment of membrane permeability increases the uptake of PI in an antagonistic sense there are no problems to distinguish necrotic from apoptotic or vital cells in a dual parameter plot.

Strong staining of cells by fluorescein as it also happens by the accumulation of the dye in this method leads to problems in anyway difficult compensation between green and red fluorescence. We suppose an auto-filtering effect of fluorescein, by what the fluorescence emission shifts more to the reddish spectral part. So it is not so easy to arrange low and very high labeled cells orthogonally in dot piots for quadrant statistics. Additionally, the apoptotic and vital cells still overlap somewhat in green fluorescence requiring oblique windows for secure discrimination. For these reasons we recommend the acquisition of green and red fluorescence without compensation.

VIABILITY ASSESSMENT OF BACTERIA BY FLOW CYTOMETRY AND SORTING AFTER FOOD PRESERVATION MEASURES

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Mild food preservation methods aim to lower the probability of outgrowth and extend the lag times of bacteria to increase product safety. A flow cytometry approach is presented to evaluate effects of single or multiple foodprocessing steps on microbial viability and to assess the degree of induced damage.
Lactobacillus plantarum, a Gram positive food spoilage micro-organism, has been chosen as the test model. Applied treatments (also in combination) were heat, the antimicrobial peptide Nisin,
the cell wall degrading enzyme Lysozyme and pH-stress.
Carboxyfluorescein diacetate, also its succinimidylester and propidium iodide were used as fluorescent molecular markers to indicate esterase activity, internal pH and membrane damage. Cells sorting has been applied to correlate with recovery, using a Coulter Epics Elite ESP flow sorter with Argon laser.
This approach turned out to be suitable
to identify and isolate reproductive treatment-survivors within the population,
- to verify the mode of preservation action on bacterial physiology,
- to assess the internal pH,
- to determine the end of the lag time and
- to follow cell proliferation.

This application of flow cytometry in food-related research was able to go beyond conventional plate-counting techniques, which give no information about the underlying mode of treatment action or the target sites within the cell.

OFF-LINE-MONITORING VON BRAUHEFEVERFAHREN

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Es werden Routineergebnisse des off-line Monitoring von Brauhefen aus verschiedenen sächsischen Brauereien vorgestellt.

Es ist üblich, Hefezellen in sogenannten Vorkultivierungsreaktoren anzuziehen, um eine für die Gärung notwendige Zellzahl zu erreichen. Es können aber auch Lagerhefen für eine erneute Führung eingesetzt werden. Ziele der Vorkultivierung sind zum einen das Erreichen einer ständigen Juvenalisierung der Kultur, wodurch deren physiologische Eigenschaften verbessert werden, und zum anderen die Verhinderung mikrobieller Kontaminationen, die während eines langen Verbleibs der Hefezellen in Lagertanks auftreten können. Im Anschluß an die Vorkultivierung werden diese Zellen (oder die Lagerhefen) zusammen mit dem Sud in die Gärtanks inokuliert (das sogenannte Drauflaßverfahren). Während dieses Verfahrens durchlaufen die Zellen noch einmal einen vollständigen Zellzyklus. Die physiologischen Eigenschaften der Zellen aus diesem Zeitraum (Wachstum unter aeroben Bedingungen) sind sehr verschieden von denen der sich anschließenden anaeroben Bedingungen der Gärung. Während der Proliferation verfügen die Zellen über einen hohen 3-ß-Hydroxysterolgehalt sowie über einen geringen Neutrallipidgehalt (Sterylester und Triacylglyceride).

Der physiologische Zustand der Hefen ist in sehr hohem Maße von der Art der Prozeßführung, die innerhalb einer Brauerei als auch von Brauerei zu Brauerei mitunter sehr verschieden gehandhabt wird, abhängig. Vorschläge zur Prozeßoptimierung werden präsentiert.

FLOWCYTOMETRISCHE BESTIMMUNG DER MEMBRANPOTENTIAL-BEZOGENEN FLUORESZENZINTENSITÄT VON *RALSTONIA EUTROPHA* JMP 134 UND *ACINETOBACTER CALCOACETICUS* 69-V

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Lebende Zellen sind fähig, in Anwesenheit einer Kohlenstoff- und Energiequelle ein Membranpotential aufzubauen. Dieses wird für bestimmte Zellprozesse, wie z. B. Stoffaufnahme- und Transportvorgänge über die Membran, benötigt. Der kationische, lipophile Fluoreszenzfarbstoff 3,3-Dihexyloxacarbocyaniniodid ($DiOC_6(3)$) konkurriert mit Kationen um die negativ geladenen Kopfgruppen der Phospholipide auf der Innenseite der Membran. Mit Hilfe eines Flowcytometers ist es möglich, an einzelnen Bakterienzellen die Membranpotential bezogene Fluoreszenz-intensität zu bestimmen. Durch solche Messungen ist es möglich, Aussagen über die zelluläre Stoffaufnahmekapazität zu treffen, insbesondere auch dann, wenn die Wachstumsbedingungen für die bakteriellen Zellen verändert werden.

Um die Färbemethode zu testen, wurden *Ralstonia eutropha* JMP 134 und *Acinetobacter calcoaceticus* 69-V als Modellorganismen eingesetzt. *Ralstonia eutropha* wuchs auf Acetat und Phenol. *Acinetobacter calcoaceticus* wurde auf Acetat bei verschiedenen Temperaturen kultiviert.

Informationen zur Teilungsaktivität der Bakterienzellen wurden durch flowcytometrische Bestimmung des DNS- Gehaltes nach DAPI - Färbung erhalten.

Detection of Circulating Fetal Cells Using Automated Fluorescent Microscopy

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Background: Fluorescent microscopy is a dynamically growing field in today's medical research and practice. As FISH (Fluorescent In Situ Hybridization) is getting more available so is the need increasing for more sophisticated methods and computer automated applications.

Aims: To make an automated system which is capable of analyzing a whole fluorescent slide without any human interaction during the process, and to develop a method to detect FISH labeled fetal blood cells in adult blood.

Materials and Methods: We analyzed 21 week cultured fetal liver blood (cFLB) spiked into adult peripheral blood (1:10, 1:100, 1:1000). All cells were counterstained with DAPI. mRNA for fetal (γ) hemoglobin was detected with an oligo probe and visualized by FITC. Staining was provided by Boehringer Mannheim. Pictures were taken from a Zeiss Axioplan 2 MOT, fully motorized microscope with a one chip color CCD camera (Grundig, Germany) and transferred to a PC with a Screen Machine II frame grabber (Fast Electronics, Germany). We used Windows NT 4.0 operating system (Microsoft) and C++Builder (Borland) for software development and the microscope is controlled trough DLLs shipped from Zeiss Germany.

Results: After thresholding the pictures to eliminate background noise, the software separates single and clustered cells. Cell center and diameter is determined. Twelve statistical parameters are calculated from hue values of each cell. These parameters are analyzed with fuzzy logic to classify fetal, adult (maternal) and trophoblast cells. A slide scanned by the microscope provides 250 microscopic images. The system can analyze one slide approximately in an hour when scanning is included (on-line mode), and in 20 minutes on pre-scanned slides (off-line mode). There are around 5000 to 7500 cells to analyze on each slide. The system can locate correctly 95% of the cells. The classification of fetal cells results in 11,07% false positive rate, only 2,81% false negative rate. Eight percent of the classified trophoblast was false positive with 6% false negativity. The relatively high percentage of false positive fetal cells was allowed to minimize the false negatives, due to the fact that maternal blood contains only very few fetal cells and only minimal loss is affordable.

Conclusions: A reliable method is found to obtain fetal cells during pregnancy by a non-invasive diagnostic method. The system we developed proved that it is possible to build a highly automated fluorescent microscope system and rare cells can be detected, located and classified optically with good efficiency. The environment around the image-processing module can be used to many different FISH applications to extend laboratory capability.

Gap Junction Communication and Cell Cycle Alters the Calcium Response to Auto/Paracrine PDGF Stimulation in A172 Glioblastoma Cells.

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Proliferation of glioblastomas involves auto / paracrine signalling via the PDGF receptor (PDGFR), which gives rise to a biphasic calcium transient of intra- and extracellular components. In A172 glioblastoma cells the complete biphasic calcium response is absent in most non-confluent (single) cells and present in 95% of confluent cells. To test the cause of this difference, the possible effect of cell-cell communication and cell cycle on PDGF evoked calcium signals was investigated by Fura-2 based dual excitation calcium imaging.Confluent cells responded to PDGF after a latency of 35s, with a peak 191% above basal level and maintained elevated [Ca2+]i for over 600s. In single cells, earliest responses were seen after 120s, and peaked only at 128% of baseline. To dissect the intracellular release phase from the influx component, thapsigargin (TG) was used for releasing Ic. calcium in place of PDGF evoked IP3 production. Ec. calcium was chelated with EGTA and Ca flux through membrane ICRAC (intracellular release activated calcium influx) channels was assessed from the peak of calcium transient upon repletion of ec. Ca. Confluent cells produced peaks of 182 % above baseline, while single cells showed no distinct peaks. We therefore suggest that confluency must influence Ca influx regulation at a level distinct from PDGFR expression or PDGF induced intracellular release. To find possible sources of this modulation we have tested its dependence on cell cycle and cell-cell communication. Using BrdU incorporation and DNA quantitation by PI fluorescence, we showed that the calcium response is independent of cell cycle. Patch clamp measurements were used to show that confluent cells exhibit significant ion fluxes among each other, which might influence calcium regulation. Lucifer Yellow dye transfer has revealed gap junction communication in confluent cells which could be uncoupled with octanol. Uncoupled confluent cells responded to PDGF after a longer latency (min. 70s) with a smaller peak and only for less than 220s. Also, uncoupled confluent cells presented significantly smaller calcium peaks upon TG stimulus. These parameters are reminiscent of those seen in single cells. Thus it is possible that the difference in the calcium responses of confluent and single cells is caused (at least) in part by gap junction communication. This could be a new aspect of the role of gap junctions in tumor growth contact inhibition.

Investigation of Chromosome Loss in Mouse-Mouse Hybridomas by Chromosome Painting

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After fusion of spleen cells and myeloma cells, the genome of hybridomas seems to be destabilized and chromosome loss occurs. Fluorescence *in situ* hybridization (FISH) of specific chromosomes (chromosome painting) is a powerful tool in cytogenetics allowing direct detection of selected chromosomes.

Here we report first results of investigation of the total chromosome numbers and the loss of chromosomes 1, 6, 12 and X of hybridomas in culture by chromosome painting. We have constructed cell hybrids between P3X63Ag8.653 mouse myeloma cells and lymphocytes derived from BALB/c mice immunized with thyroglobulin. Three hybridomas (A4, D8, F10) were selected and after one cloning the cells were cultivated *in vitro* over a period of 28 days. After a number of passages (1, 2, 3, 9) air-dried metaphase spreads were prepared by standard method and for FISH digoxigenin- and biotin-labeled mouse chromosome painting probes (Oncor) and rhodamine-anti-digoxigenin and fluorescein-avidin for dual color detection were used.

Mean chromosome numbers of 78 (D8), 82 (F10) and 150 (A4) were observed. Theoretically, the hybridomas should contain a total of 91 chromosomes (40 chromosomes from mouse spleen cells and 51 from myeloma cells). The total number of chromosomes in hybridomas as well as the number of chromosomes 1, 6, 12 and X did not significantly decrease between the first and the ninth passage, however, a lot of chromosome rearrangements were detected in comparison with the parental cells.

Our results demonstrate that the rearrangement and stabilization of the hybridoma genome probably occurred during the initial first cell cycles.

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12th Heidelberg Cytometry Symposium

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Annual Meeting of the German Society of Cytometry

21st to 23rd October 1999

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