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Abstracts

Oral presentations (alphabetical order)

Interferon-g- beeinflußt den Apoptose-Signalweg von TNFa nur teilweise über NFkB in der humanen Tumor Zell-Linie Me-180

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In Me-180 Zellen induziert TNFa Apoptosis. Die Zellen lösen sich vom Kulturflaschenboden und zeigen apoptotische Charakteristika wie Chromatin-Kondensantion, Mitochondrien Membran Depolarisation, Phosphatidyl-Serin Exposition, PARP-Spaltung. Interferon g (IFNg) induziert fast keine Apoptosis, die Kombination von TNFa und IFNg ergibt jedoch einen dramatischen Anstieg des Anteils apoptotischer Zellen. Das deutet auf "crosstalk" zwischen den TNFa- and IFNg-Signalwegen hin. Durch TNF werden pro- und anti-apoptotische Signale ausgelöst. Ein anti-apoptototischer Weg führt über den Transkriptionsfaktor NFkB. Vom IFNg ist bekannt, dass es über STAT-1 die Expression von Inhibitoren (z.B. p202) der Transkriptionsfaktoren AP-1 and NFkB aktiviert. Dies könnte als Mechanismus, der die TNF-induzierte Apoptose durch IFN verstärkt, in Frage kommen.

Wir haben die NFkB Veränderung im Zellkern nach TNF und IFN untersucht. Mit Antikörpern gegen NFkB haben wir eine kurzfristige Erhöhung nach TNF und Absenkung nach IFN nicht gefunden. Allerdings deutete ein Abbau von ikB nach TNF auf eine Aktivierung von NFkB hin. Dies wurde mit transienter Transfektion von Plasmiden, die für ikB-Grün-Fluoreszenz-Protein Fusionsprotein kodierten, gemessen. Nach 1 bis 3 Tagen TNF- Einwirkung war NFkB im Zellkern etwa doppelt so hoch wie in der Kontrolle, während IFN keine Veränderung gegenüber der Kontrolle bewirkte. Durch die gemeinsame Gabe von IFN und TNF konnte der NFkB-level nicht gegenüber TNF allein abgesenkt werden. Diesen nicht ganz dem Modell entsprechenden Ergebnisse stehen Resultate aus Messungen mit NFkB-Inhibitoren gegenüber. Mit einer Reihe verschiedener Inhibitoren konnte durchgehend der Apoptose-Anteil von TNF allein erhöht werden, wenn sie in Kombination mit TNF gegeben wurden. Allerdings wurde in keinem Fall eine so starke Steigerung der Apoptose wie durch IFN erreicht. Insgesamt zeigen unsere Ergebnisse, dass NFkB in dieser Zelllinie nur zu einem geringen Teil der Wechselwirkung zwischen den IFNg- und TNFa-Signalwegen zu Grunde liegt.

Detection of Membrane Glucocorticoid-Receptor Expression on Peripheral Blood Monocytes Using High-Sensitivity Immunofluorescence

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Glucocorticoid receptors (GCR) can easily be detected by conventional immunofluorescence in the cytoplasm but not on the surface of many different cell types. Recent data provide evidence that GCR are expressed at low level on the surface of certain leukemia cell lines. We have used high-sensitivity immunofluorescent staining (magnetofluorescent liposomes) to analyze the expression of GCR on cell lines and normal peripheral blood mononuclear cells (PBMC). We compared the frequency of GCR positive cells in PBMC from vaccinated persons, Rheumatoid Arthritis patients, and healthy controls. Basic GCR expression (5-6%) could be demonstrated on monocytes from healthy controls. However, healthy people following vaccination and patients with active Rheumatoid Arthritis have clearly elevated level of GCR expression on peripheral blood monocytes (up to 20%). The expression level also correlates with clinical parameters of disease activity.

This data clearly demonstrate GCR surface expression on normal human peripheral blood monocytes. Furthermore the expression level seems to correlate with an activated immune system, e.g. in chronic autoimmune disease or following vaccination.

Cell Cycle and Growth Response of CHO Cells to Low Doses of X-Rays

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This study was stimulated by reports of hyperradiosensitivity (HRS) of various cell lines to very low X-ray doses. HRS could have implications in both, clinical and environmental exposure. It has be en interpreted as a result of a threshold for the induced repair (IR) of DNA damage.

Survival data of exponentially growing X-irradiated Chinese hamster ovary cells were acquired by conventional colony forming assay and by flow cytometric population counting. The data were subjected to best-fit analyses with both, the linear-quadratic (LQ) and the IR model. Flow cytometric DNA and DNA/BrdU measurements served to test cell cycle reactions for compatibility with HRS.

Both, the LQ and the IR model sufficiently describe survival curves established by colony forming assays. In contrast, population counting data were exactly and exclusively in accordance with the LQ model. Four hours after irradiation, cell cycle distributions showed a dose dependent mitotic block, delay of cells in G2 and, after higher doses, increasingly in late S-phase. All alterations were monotonous between 0.2 and 3 Gy.

No convincing evidence of HRS was found in these experiments. For survival data, the LQ model applies to the full dose range from 0.2 to 7 Gy. The sensible cell cycle response is incompatible with an "ignorance" of the cells even to low amounts of DNA damage. More likely, they reflect an alert condition, supporting the assumption of a threshold-free IR.

Multiparameter Analysis of Progeny of Individual Cells by Laser Scanning Cytometry (LSC)

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LSC was adapted to analyze size and phenotype of colonies of MCF-7 cells growing in microscope slide chambers, untreated and treated with the cytotoxic ribonuclease, onconase (ONC). Data representing each colony was segmented based on BODIPY 630/650-X fluorescence (> 650 nm) excited by a He-Ne laser. The cells' DNA stained with PI and estrogen receptor (ER) or p53 (FITC) were detected by Ar ion laser excitation. The following attributes of individual colonies were measured: (a) area, circumference, area/circumference ratio, (b) DNA or protein content/colony area, (c) number of cells (nuclei), (d) DNA content and cell cycle distribution, (e) protein content and protein/DNA ratio, and (e) expression of ER or p53 per colony, per total protein, per nucleus or per DNA, within a colony. Heterogeneity of colonies with respect to all the measured features was estimated. Changes in colony size and phenotype reflecting altered cell shape, size, colony protein/DNA ratio, expression of individual proteins, may reveal mechanisms of drug action to suppress proliferative capacity of the cells, and detect growth imbalance, differentiation and modulating expression of the genes that may be associated with cell cycle, apoptosis or differentiation. With minor modifications LSC may be applicable for automatic analysis of cloning efficiency and multiparameter analysis of cell colonies in soft agar. Such analyses may be useful in studies of mechanisms and effectiveness of antitumor drugs, in field of carcinogenesis, for analysis of primary cultures, tumor prognosis and drug sensitivity and in analysis of microbial colonies.

Apoptosis and Caspase Activity in Peripheral Blood Lymphocytes of Patients with Lupus Erythematosus Detected by Flow Cytometry

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Cells undergoing apoptosis can be detected flow cytometrically be a variety of different methods including annexin V assay, disruption of mitochondrial membrane potential (??m), TUNEL Assay, detection of

poly(ADP)-ribose-polymerase fragmentation, and sub-G_1-peak for example. Sequential activation of initiator and effector-caspases (cysteine proteases with aspartic acid specificity) is a biochemical hallmark of cells undergoing apoptosis. Commonly used techniques for the detection of activated caspases in different cell systems are western blot analysis, colorimetric and fluorometric assays. These are time consuming methods and additionally have the disadvantage that a simultaneous labelling with specific mAbs is not possible. To overcome these problems in the present preliminary investigation we used covalently to rhodamine 110 [$(L\text{-}Asp)_2$-rhodamine 110] bound aspartyl compounds as pan-caspase substrate. The complete complex is colorless, but after caspase-induced cleavage the fluorochrome is released and can be detected either by flow cytometry, fluorescence microscopy or laser scanning microscopy (excitation 488 nm, emission 515 - 545 nm). Since patients with lupus erythematosus display an increased in vivo frequency of blood cells undergoing apoptosis, we analyzed peripheral lymphocytes of these patients. Freshly obtained whole blood samples were compared to density gradient centrifugation isolated PBMC. Additionally, apoptosis was measured by using the annexin V assay.

We could find caspase activity in peripheral lymphocytes of patients with LE as compared to healthy controls with this technique. $(L\text{-}Asp)_2$-rhodamine staining was possible in PBMC as well as in whole blood samples. Annexin V Labelling of membrane phosphatidylserine exposure did not correlate with $(L\text{-}Asp)_2$-rhodamine cleavage. Taken together, the obtained results show that $(L\text{-}Asp)_2$-rhodamine 110 is an interesting tool for the flow cytometrically based investigation of pan-caspase activity, because (i) it is a rapid method suitable to screen pan-caspase activity on single cell level, and (ii) it can be used with simultaneous mAb staining. Further investigations to confirm this observation and clarify it in more detail are currently underway.

Objective Grading and Prognostification of Dysplasias of the Uterine Cervix Using a Combination of Laser Scanning Cytometry and HPV-PCR. An one Step Method.

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Grading of cervical dysplasias as a morphological method is subjective. DNA aneuploidy is regarded as the starting point of cervical carcinogenesis. Aneuploidy can be measured by interactive image cytometry after Feulgen staining. Image DNA-cytometry of pap-smears after Feulgen staining has proved as an objective method of classification: DNA diploid dyplasias are low risk lesions (LSIL), DNA aneuploid dysplasias are high risk lesions (HSIL). Image cytometry though only can be done as a second step next to screening the stained pap-smears to render a diagnosis. 1The newly developed Laser scanning cytometer (LSC) in combination with 1fluid cytology makes it possible to perform simultaneously cytological diagnosis, DNA ploidy measurement and HPV-typing .

Material and methods:

From 22 patients with known SIL ("III-D") we performed monolayer preparations using the ThinPrep-Paptest (Cytyc). DNA ploidy was measured after PI staining with the LSC (Compucyte). Subsequently the smears were stained according to Papanicolaou and visualisized and classified after automatic relocalisation regarding to the DNA content und coordinates. Simultanously we performed detection and typing of HPV by PCR and direct sequencing.

Results:

Results of simultaneous DNA-cytometry of ThinPrep-Preparations using the LSC (Compucyte) and HPV-typing by PCR in 22 cases with squamous intraepithelial lesions of the cervix uteri:

Ploidy HPV-Type

	negative	low risk	high risk	unknown risk	no typing possible
diploid	1	-	6	-	2
polyploid	-	-	5	-	-
aneuploid	-	-	7	1	-

Conclusion:

Our results indicate that laser scanning cytometry of fluid cytology in combination with HPV-PCR lead to an objective morphological and molecular grading and prognostification of cervical dyplasias in one step.

DNA-Image Zytometrie in der Subtypisierung der Grosszell- Lymphome

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Das Tumoren Wachstum ist mit den Veränderungen in dem DNA Inhalt- Aneuplöidie karakteriziert. Die morfologische Kennzeichnungen der Tumorzellen, die in der May-Grünwald-Giemsa gefärbten Preparaten erkennbar sind, sind mit der Hiperkromasien des Zellkerns karakteriziert.

Mit dem automatischen Bildanalizierung System, das aus einem Mikroskop, einer Video Kamera und einem Computer mit dem speziellen Software besteht, können die Kromatinfärbung Intenzität Veränderungen numerisch objektiviziert werden.

Wir haben 22 Aspiratzion Punktate der Lymphknoten analiziert – 4 Patienten mit dem Burkitt oder Burkitt-like Lymphom, 6 Patienten mit dem Anaplastisch Großzell Lymphom (ALCL) und 12 Patienten mit dem Diffus Großzell-Lymphom (DLBCL). Die Aspiration Material Ausstriche wurden mit der Feulgen Methode gefärbt.

Die Ergebnisse haben die Heterogenität des DNA-Inhalts in dieselbe Lymphom Gruppe gezeigt. Mehr als 30% Zellen in der S+G2/M Phase oder mehr als 30% Zellen mit DNA>4N sind mit dem schnellen Relaps und mit der kürzeren Überlebung verbunden. Die richtige Quantisierung des DNA- Inhalts spielt eine Rolle nicht nur bei der Diagnostizierung sondern ist auch wichtig für die Klasifizierung und die Prognose besonders der bösartigen Tumoren.

Cytometric Approaches To The Molecular Analysis of Cytokine Memory

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By expression of cytokines, T helper lymphocytes control immune reactions and contribute to immunopathology. To investigate the molecular basis of cytokine expression of individual T cells, we have developed and used various cytometric and cell sorting technologies.Using the cytokine secretion assay for the isolation of T cells expressing distinct cytokines, like interleukin-4, interleukin-10 or g-interferon, we could show that Th cells, upon restimulation by antigen, recall the expression of those cytokines for which they had been instructed in earlier activations. Cells isolated according to their expression of interleukin-4 show high expression levels of the transcription factors GATA-3 and c-maf. Exogenous overexpression of GATA-3 in Th cells induces their differentiation into Th2 cells, which are characterized by the expression of Il-4, Il-5, Il-10, and Il-13. Further, the expression of endogenous GATA-3 was induced by exogenous GATA-3, revealing an autoregulatory loop controlling GATA-3 expression levels and , thereby, Th2 differentiation and memory. To analyse GATA-3 expression in individual Th2 cells in the course of their differentiation, we have established a protocol to detect GATA-3 intracellularly by immunofluorescence.

Models of Microevolution: Opposing Mechanisms of Tumour Cell Survival after Genotoxic Treatment

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The relationships between mitotic death, development of giant cells, delayed apoptosis, and survival displayed by p53 mutated tumours, are currently unclear. To study them, two sets of parental lymphoblastoid cell lines were used: TK6 and WI-L2-NS (wt and mt p53, one point mutation), and two diverged laboratory strains of the Namalwa cell line, N99 and N84 (two and three p53 point mutations), respectively. These were assessed over a 3 week period after single doses of external beam irradiation ranging from 2 to 15 Gy using DNA flow cytometry, quantitative 3H-T-radioautography, clonogenic assays, and counts of mitosis. The radioresistance of the cell lines was in the increasing order TK6, N99, WI-L2-NS and N84. Clonogenicity positively correlated with the irradiation dose causing the maximum number of cells to arrest in the G2 compartment, on the first day post-damage. The delays and apoptosis from G1 and S-phase checkpoints were different in the two models of microevolution: strong delays from both in TK6; no G1- but some S-phase delay in WI-L2-NS; G1- but no S-phase delay in N99; absence of both checkpoints in N84. The kinetics of delayed apoptosis, resumption of mitosis, and formation of endocycling giant cells indicated to a similar origin from the extended G2-arrest. All cultures retained strong spindle checkpoints as seen by apoptosis of aberrant metaphases. The complete absence of normal mitoses followed by interruption of the mitotic pathway, after 10-15 Gy in Namalwa cell lines, indicated insufficient DNA repair in the pre-mitotic compartments. However, in these cases the giant cells underwent complex changes and segregated into survivors reconstituting the mitotic pathway. In all other

instances, the wt and mt p53 tumours recovered from the descendants of the non-interrupted mitotic

pathway, while giant cells degraded. Conclusions: There exist two opposing mechanisms of surviving genotoxic damage in p53 mutated cell lines -via the mitotic cycle or via transient endocycles. Importantly, the spindle checkpoint is not eroded by tumour progression in either model.

Cell Cycle-Related Changes in the Fluorescence Lifetime of Ethidium Bromide Associated with Alterations in Chromatin Structure in Mammalian Cells

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DNA-binding fluorescent dyes have been employed for more than two decades to determine cell cycle distributions using flow cytometry. These analyses are possible, because the fluorescence intensity of a number of DNA binding dyes is approximately proportional to DNA content in fixed cells. Whereas this is well established, we wondered whether changes in nuclear organization or other factors might subtly alter the fluorescence properties of dyes bound to DNA in cells from different parts of the growth cycle. To investigate this possibility, we used multiparameter flow cytometry to measure the fluorescence lifetimes and the fluorescence intensities of individual cells stained with the DNA-binding dyes, ethidium bromide (EB) or propidium iodide (PI), in conjunction with other cellular parameters.

Bivariate plots of fluorescence lifetime and fluorescence intensities of asynchronous human skin fibroblasts stained with EB revealed a small 5% sub-population of cells near the G1/S boundary whose average EB fluorescence lifetime was reduced, compared with average lifetimes in the rest of the cells. In contrast, the sub-population was absent in cells stained with PI. Simultaneous measurement of G1 cyclins using FITC-labeled antibodies demonstrated that the decreased EB-lifetime sub-population contained elevated levels of cyclin D1 and Cyclin E, confirming its fluorescence-intensity-assigned location near the G1/S boundary. In addition, total cellular proteins were elevated in the reduced EB-lifetime sub-population.

Synchronization of cells in early S phase by treating serum-stimulated G0 cells with aphidicolin increased the portion of cells having the reduced EB-lifetime. Following the release of cells from the aphidicolin block, the EB-lifetimes increased as total cellular protein decreased and cells progressed into the S phase. These novel studies demonstrate that changes in EB dye binding are correlated with changes in cyclin/protein expression and, putatively, a nuclear/chromatin structural transition near the G1/S boundary. As such, they indicate the potential of multiparameter flow cytometric analysis to identify changes in DNA accessibility or nuclear/chromatin structure during the cell cycle.

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Flow-Cytometric Analysis of TGF-? Producing T Cells

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TGF-? is a cytokine with multiple functions *in vivo*. It is produced by a variety of different cell types and has been shown to play an important role as an anti-inflammatory cytokine and for downregulation of immune responses. TGF-? has been described as the main effector molecule produced by regulatory or suppressor T cells, which are induced e.g. by oral tolerogenization. Despite multiple reports showing the important role for TGF-? produced by T cells for immune suppression no direct evidence for the existence of a discrete population of TGF-? producing T cells has been provided. We have established intracellular staining protocols for the different subunits of the intracellular form of TGF-?. Intracellular staining of activated T cells revealed that the maximum expression can be observed after 48 hours of stimulation with antigen or CD3/CD28. These kinetics are in accordance with ELISA data and the maximum expression of CTLA-4 a negative regulator of T cell activation, which has been shown to function as an inducer of TGF-? production *in vitro*. More interestingly at this timepoint all T cells seem to express TGF-? at a low level. This is in striking contrast to most other T cell cytokines, which are strongly expressed only by certain T cell subpopulations. This coordinate expression of TGF-? at late timpoints of T cell activation might reflect a general autoregulatory reaction of all T cells rather than the existence of a defined TGF-?-producing regulatory T cell subset.

Differential chemokine expression by T cell subsets

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Functional T cell subsets have been defined according to the expression of distinct cytokines. Recently the differential expression of chemokine receptors in these T cell subpopulations has also been reported, implicating an important link between T cell effector function and the migratory capacity of these cells. However, up to now only few data are available about the expression of chemokines in T cell subsets. We have established intracellular FACS staining for the classical chemokines MIP-1?, MIP-1?, RANTES and MIP-2 and for the chemokine-related protein ATAC. We found that these molecules are exclusively expressed in memory, but not in naive T cells. Furthermore MIP1-?, MIP1-?, RANTES and ATAC are mainly expressed in *in vitro* differentiated Th1, but not Th2 cells. In T cells polyclonally activated *ex vivo* the expression of these chemokines is positively correlated with IFN-?, the key Th1 cytokine. In contrast MIP-2 is exclusively expressed in Th2 cells.

These data show for the first time on the level of single cells the strongly polarized expression of chemokines in T cell subsets. In fact, the selective expression of certain chemokine groups might play an important role for the recruitment of appropriate effector cells of the adaptive and innate immune system to the microenvironment and might therefore influence the efficiency and outcome of an immune response.

Assembly of the Nuclear Envelope Membrane Analyzed in vivo by Quantitative 4D Image Reconstruction

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Higher eukaryotic cells break down their nuclear architecture during prometaphase of every cell division. The first step to reconstitute nuclear organization after mitosis is the reassembly of the nuclear envelope (NE) around chromosomes. This dynamic process can be monitored in single living cells in three dimensions over time by multi-color GFP confocal time-lapse microscopy. Such 4D sequences of Lamin B receptor fused to a yellow spectral mutant of GFP (LBR-YFP) and Histone 2B fused to cyan variant (H2B-CFP) were acquired, allowing us to simultaneously visualize the inner nuclear membrane and chromatin in dividing cells. We have developed image analysis tools to visualize and quantitatively analyze 4D imaging data: Fully automated image segmentation and interpolation techniques were applied to reconstruct three-dimensional surface models from image stacks at individual time sections. A continuous reconstruction over time was carried out by interpolating morphing algorithms.

LBR-YFP is distributed homogenously throughout the cytoplasmic ER after NE breakdown in prometaphase. Three-dimensional reconstruction of the NE showed that reformation of the NE occurs in two phases: Distinct membrane patches enriched in LBR-YFP form on the chromatin surface in late anaphase. These initially tubular structures expand to sheets and later fuse simultaneously as well as symmetrically in both daughter nuclei to completely enclose the chromatin. The concentration of LBR-YFP in these areas of contact with chromatin increases rapidly 2.4 fold over that in the ER indicating binding of the protein to the chromatin surface. The surface of NE and chromatin appears highly invaginated at this stage. Then, NE and chromatin both expand, whereby the surface smoothes. Volume measurements revealed that chromatin and NE expand with similar rates, indicating that NE expansion is directly driven by the decondensation of chromatin.

Ligand Specific Heteromeric CD14-Clustering in Inflammation

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The monocyte specific glycosylphosphatidylinositol-anchored endotoxin receptor CD14 plays a major role in the inflammatory response to lipopolysaccharide (LPS) without transducing signals by itself. Recently, additional ligands have been detected suggesting a pleiotropic function. Here we describe that ceramide, a constituent of atherogenic lipoproteins, acts as extracellular ligand for CD14. LPS and ceramide receptor-ligand interactions were compared based on clustering of CD14 to co-receptors. Both, LPS and ceramide induced co-association of CD14,

complement receptor 3 (CD11b/CD18) and CD36. Interestingly, only LPS induced co-clustering with Toll-like receptor 4, Fc?-RIIIa (CD16a) and the integrin associated protein (IAP) CD81, whereas ceramide induced clustering with CD47, another IAP. This suggests the existence of two different cellular pathways that are linked to the assembly of innate receptor complexes in rafts. LPS docking to CD14 is accompanied with the release of proinflammatory mediators in sepsis, and ceramide binding to CD14 is associated with atherosclerosis.

Analysis of Single Molecules

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The reaction catalysed by single or a few enzyme molecules can be studied by generating femtodroplets of enzyme solution containing such a small number of enzyme molecules. After fusion of this droplet with a similar droplet containing substrate solution, the reaction starts. In cases where the nonfluorescent substrate is converted into a fluorescent product, the reaction course can be observed via the increase of fluorescence. In a second type of single molecule experiments an individual DNA molecule is coupled to a polystyrene microsphere and can then be handled with optical twweezers. The biomechanics of a single DNA molecule is studied by stretching the molecule and suddenly allow it to collapse into a globular form. Also, rstriction analysis is possible and individual DNA molecules can be characterized similarly as this is done in bulk by RFLP analysis. Thereby it becomes evident, that restriction endonucleases have a surprisingly high processivity.

Absolute Counting of Low Level Leucocytes on Leucocyte Depleted Blood Products

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Residual white blood cells (WBC) in blood products, such as red blood cell concentrates or platelet concentrates, have been shown to trigger transfusion-related complications in the recipient of the blood products. Therefore a WBC-counting technique is required that can reliably measure very low cell concentrations. The leucocyte numbers are below the range detectable of automated cell counters and have been conventionally determined with light microscopic visualisation in the Nageotte counting chamber. This is a time-consuming and laborious technique. We here describe a sensitive and rapid method for counting low leucocyte numbers using a flow cytometer. DAPI (4´-6-diamidino-2-phenylindole) stained leucocyte-DNA was measured using a flow cytometer with UV-lamp and cell concentration was calculated automatically by volumetrical counting. The detection limit of the method is 0.5 leucocytes/µl whole blood sample diluted in buffer, red blood cell or platelet concentrates. This threshold is far below the required sensitivity for quality control in Transfusion Medicine. The technique is proved to be highly accurate and allows an inexpensive routine quality control of blood products.

Chromatin Condensation During Apoptosis Correlates with the Release of Cytoskeletal Tension on the Cell Nucleus

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Using 4-D microscopy, we investigated the dynamic reorganization of chromatin in live cells undergoing apoptosis. Following these cells in real time we see a progressive correlation of the release of cytoskeletal tension as the cell detaches from the growth surface and the extent of chromatin condensation during apoptotic progression. In order to determine whether or not cytoskeletal tension is the major modulator of interphase chromatin structure, we also examined cells undergoing trypsinization to release them from the growth surface. Although the nucleus and chromatin appear to undergo some degree of condensation during this process, the released cells do not adopt spherical chromatin bodies. This indicates that an alteration in nucleus-chromatin interactions, which likely precede the final condensation events, are essential for the full apoptotic phenotype. By examining cells undergoing cell spreading in early G1, we observe a strong correlation between chromatin decondensation and cell tension. We conclude that the native state of interphase chromatin is to adopt a condensed phenotype and that it is held apart by cell tension that is transmitted to the nucleus. This differs from the classical biochemical viewpoint that processes such as chromatin remodeling and histone posttranslational modification drive the decondensation of chromatin during interphase.

Assessment of Grade in Follicular Lymphoma: Correlation Between Light Scatter Properties and Histologic Appearance.

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Flow cytometry is a rapid and sensitive means of characterizing hematolymphoid malignancies, with immunophenotype and cell cycle analysis both offering diagnostic and prognostic information. Although light scatter properties are typically used to define normal cell subsets and to determine gating strategies, they are underutilized as a means of characterizing malignancies. This lecture presents results of a study designed to test the feasibility of using light scatter properties as an added diagnostic parameter. The model studied is follicular lymphoma, conventional grading of which relies on microscopic examination of tissue sections: the higher the number of large cells within the tumor, the higher the tumor grade. Details of the flow cytometric analysis will be presented, and the results correlated with initial histologic grading (at time of diagnosis) and retrospective histologic grading (by consensus).

T-Gamma-Lymphoproliferative Disease - A Case Report

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As one special clinical and pathological entity inside a large group of lympho-proliferative diseases, T-gamma lymphoproliferative disease (T-g-LPD) is cha-racterized by a neoplastic clone of T-gamma -lymphocytes that very rare infiltrates bone marrow or / and peripheral blood, but often developes in a chronic disease with recurrence infections.

In January 1998, because of recurrence infections, high body temperatures and sore throat for a long period of time (about one year), a patient (aged 31)

came for an out-patient treatment to the Department of Internal Medicine University Hospital "Merkur". Laboratory tests showed a normal counterpart of the white blood cells (WBC = 6.9´ 109/L; Hb =147g/L; PLT = 203´ 109/L), but absolute lymphocytosis (Ly = 5.3 x 109/ L) and neutropenia were found (Seg = 0.6 x 109/ L). The diagnosis of an lymphoproliferative syndrom was established, the patient released and send to communicable-disease clinic to see an epidemiologist. In June 1998, a complete medical ambulance examination (checkup) was performed including histology, serology, cytology of bone marrow aspirate, immunophenotyping of the bone marrow (BM) and peripheral blood (PB) cells together with ultrasound of the abdomen. In bone marrow and peripheral blood smears (Ly=76% and 29%, respectively) the infiltration with a neoplastic clone of T-gamma-multiplied lymphocytes containing azurophilic granules in their cytoplasm was found. Results of flow cytometric immunophenotyping of PB and BM samples proved the T-origin of the cells and gave the next profile of the T-lymphocytes: CD2+CD3+CD7+CD8+CD4-CD57+CD38+CD56- with aberant CD5 membrane marker expression (positive on 65% mature T-lymphocytes) and dominancy of cytotoxic-suppresor T-lymphocytes (CD3+CD57+; CD3+CD8+>>CD3+CD4+). The sum of T-subpopulations (T-helpers and T-inducers) was much lower than the measured positivity of CD3 membrane marker (CD3+=90.4%). Ultrasound of the liver and spleen showed no morphological changes in their structure. In kariogramm of the BM no clonal excess was found. Histology described a normocellular BM with multiplied but mature cells of lymphatic origin and do not confirm positive cytology and immunophenotyping results. Although, the majority of T-g-LPDs are cytological and morphological homogenuous, the reduced sum of mature T-subpopulations could assume the existance of more "atipical" T-g-lymphocytes inside the vide group of T-g- LPD.

Lymphocyte Subsets in Lumbar Cerebrospinal Fluid (CSF) of Aging Humans

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Introduction: In aging humans immune system exhibits reduced responses against antigens and alterations of humoral immune response. The question arises if these systemic dysregulative processes alter the immune surveillance of human central nervous system (CNS). Recent studies with adult controls showed a particular pattern of lymphocyte transfer from blood into CSF: **i.** transfer of T cells was preferred, **ii.** transfer of NK cells and B cells was barred, **iii.** transfer of immature T cells ($CD4^{+}8^{+}$) was facilitated.

Material and methods: Criteria of selection of CSF from patients (age 25 - 79 years) were: leukocytes counts (0.5-4.5 cells/ml), erythrocytes <200/ml, and total protein, Llactate, QAlb, IgG index ranging within normal values. Flow cytometric analysis of lymphocytes was done with concentrated CSF samples by adding

5 ml of monoclonal reagents, 10 ml LDS 751 working solution and 250 ml phosphate buffered saline. Data acquisition and analysis were done using FACScan-Research software (Becton Dickinson). Statistics: Effect of age (x; years) on the parameters (y) was calculated with the linear regression equation $y = a + bx$ (Pearson) and its significance p (Spearman).

Results and discussion: Number of $CD3^{+}$ T and HLA DR^{-} $CD3^{+}$ T cells decreased in CSF with age. From the lymphocyte subsets studied, number of $CD3^{+}4^{+}$ cells decreased significantly with age whereas other subsets did not: $CD3^{+}8^{+}$ T, $CD3^{+}$ HLA-DR^{+} T, $CD3^{+}16^{+}56^{+}$ T, $CD4^{-}8^{-}$, ¡ / 0 T, a/ß T, NK cells ($CD16^{+}56^{+}3^{-}$), $CD8^{+}3^{-}$, $CD19^{+}3^{-}$.

Conclusion: Our data show a reduction of $CD3^{+}4^{+}$ T and HLA-DR^{-} T cells in CSF with increasing age pointing to an altered immune surveillance in CNS of aging humans with respect to cellular and humoral immune responses.

Digital Confocal Imaging as an Alternative to Traditional Laser Scanning Techniques for Analysis of Intracellular Topography

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Aim: A digital confocal imaging (DCI) technique was applied to assess the quality of localizing intracellular receptor molecules. The biological question addressed was the topography of the cytokine receptor molecules p75Tumor Necrosis Factor (p75TNF) and its isoform icp75TNF.

Methods: HeLa cells were transiently transfected with expression plasmids encoding myc-tagged p75TNFR or icp75TNFR. Mitochondria and myc proteins were fluorescence labeled using standard techniques. Fluorescent optical sections (n=70 for each color) were obtained with a conventional Zeiss Axiovert microscope equipped with a piezoelectric z-axis focus device. Images were obtained with a Princeton Instruments charge coupled device (CCD) camera (4096 levels of gray, -15°C peltier cooled) and processed by MetaMorph software (Universal Imaging Corp.). The light haze contributed by fluorescently-labeled structures located above and below the plane of optimal focus was mathematically reassigned to its proper places of origin (EPR, Exhaustive Photon Reassignment software, Scanalytics, Massachusetts) after accurate characterization of the blurring function of the optical system (point spread function, PSF). During restoration, EPR used the PSF image data to refocus light and haze in the raw specimen image.

Results: Digital confocal images show a significant increase in contrast and sharpness due to decrease of haze and blurring, leading to an advanced resolution. Expression of p75TNFR is on the cell surface, icpTNFR is localized in intracellular compartments.

Conclusions: In contrast to confocal laser scanning microscopy (CLSM) images, DCI is based on the present PSF, thus leading to a even better correction for all optical aberrations. EPR deconvolution is a valuable alternative to real confocal techniques for photobleaching resistant samples, only.

Migration and Cytokine Expression of Monocytes in Fibroblast Spheroids Determined by Flow Cytometry

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Background and Aim: Tumor-associated macrophages (TAM) are often characterized by a lack of functional activity. They derive from circulating blood monocytes (MO) by migration and differentiation processes determined by the tumor microenvironment. In scirrhous breast tumors TAM are primarily located in the tumor-associated stroma. The objective of the present study was to investigate the impact of tumor-associated stromal fibroblasts on

the migration and function of MO. Methods: Multicellular spheroids (MCS) of fibroblasts of different origin with defined sizes were generated and monocyte suspensions were added. After defined time intervals, cocultures were dissociated and cells were stained for multiparameter flow cytometry to determine the migrated MO population and their functional status by measuring spontaneous cytokine expression. Cytokine secretion was analysed by ELISA; immunohistochemistry

was applied to visualize cell surface antigens in cocultures.

Results: In contrast to MCS of fibroblasts outgrown from normal tissue, fibroblasts from ductal carcinomas were excessively invaded by MO. A linear correlation between number of infiltrated MO and number of MO applied per MCS was shown indicating a distinct migratory MO subpopulation (» 15% of PBMC). In parallel, a 5- to 10-fold enhanced expression of cytokines (MCP-1, IL-6, IL-8) and ICAM-1 (CD54) in tumor-derived as opposed to normal fibroblasts was documented. Within MCS of tumor-derived fibroblasts, expression of some macrophage differentiation markers such as MAX3 were induces after 2 days in coculture. However, in contrast to earlier studies with bladder cancer MCS, spontaneous production of TNF-a and IL-6 within the same time interval was lacking.

Conclusions: Our data suggest that tumor-associated fibroblasts play a critical role in the recruitment of blood MO into tumor tissue. They produce cytokines and cell adhesion molecules that can induce monocyte migration and may also affect the functional maturation process of the migrated MO population. (Supported by the DFG and by the Bayerische Staatsministerium für Unterricht, Kultus, Wissenschaft und Kunst)

Analysis of Peripheral Blood Leukocytes by LSC with Sequential Staining of CD-antigens

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AIM: Immunophenotyping of peripheral blood leukocytes (PBLs) by laser scanning cytometry (LSC) is a very promising alternative to established technologies such as flow cytometry. In the LSC cells are immobilised on a glass slide and are scanned by two lasers. In addition to the fluorescence parameters the x- and y-co-ordinates of each cell are stored. This allows to re-localise every single cell at any time after finishing the analysis. We developed a novel method to use this unique feature in order to analyse the same cells a 2nd time by re-staining with a different set of antibodies.

MATERIAL & METHODS: Peripheral blood is drawn in EDTA syringes, erythrocytes are lysed, and PBLs are stained for a 1st set of CD-antigens by direct immunostaining. DNA is stained with 7-AAD. Analysis in the LSC is performed with 7-AAD as the trigger. Following this 1st analysis the cover slip is removed and cells are stained for a 2nd set of CD-antigens. The slide is analysed again. The data of both scans are merged on a cell-to-cell basis. Data obtained by this way of re-staining are compared to those gained by direct staining. Various antibodies were tested (e.g. against CD3, CD4, CD8, CD14, CD19, CD45).

RESULTS: The quality of re-staining differed for the tested CD-antigens. T-cell-antigens turned out to be very stable without detectable loss of resolution. In contrast, CD14 and CD45 show substantial decrease after re-staining as compared to direct staining. CD19 was intermediate. Type and duration of fixation were crucial steps and limited the application to certain antigens. We tested this re-staining assay with different antibody combinations. Although for some antigens 2nd step staining reduced resolution the percentages of stained cells were apparently not affected. Using antibodies with identical fluorochrome labels in the 2nd step as in the 1st step did not lead to substantial loss in the capability to detect cells. After staining, cells could be re-stained by standard H&E and subsets were re-localised to evaluate the morphology.

CONCLUSION: This new method opens the opportunity to make full use of the capacities offered by the LSC. It will allow to characterise specific cell types more thoroughly than before in a way that is only possible with multi-laser flow cytometry. However, this still lacks the opportunity to visualise the morphology of the cells.

Analysis of Fine Needle Aspiration Biopsies from Head and Neck Cancer by LSC

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Background: Fine needle aspiration biopsies (FNABs) are an important diagnostic tool for malignancies in the head and neck region. Most tumours of the oro- / hypopharynx and of the parotid as well as suspected lymph node metastases are feasible for FNABs. Analysis of cytological samples by laser scanning cytometry (LSC) offers the

unique opportunity to visualise every single cell in the microscope at any time during or after analysis; nevertheless, up to 1,000 cells are analysed per minute. We introduce a method to apply this technology on FNABs from head and neck cancer.

Material & Methods: FNABs were taken immediately after surgical resection from the primary tumour, the metastases, and disease free lymph nodes. Routine histology was performed. FNABs were placed on a glass slide, fixed, and stained by FITC-anti-Cytokeratin antibody and PI. LSC analysis was triggered on the DNA-signal. Tumour cells were identified as Cytokeratin^{+} cells. Following analysis the coverslip was removed, cells were stained with haematoxilin-eosin, and re-localised in the LSC in order to set the diploid reference peak without any doubt even when no specific Cytokeratin-signal was obtained.

Results: 7 patients with hypopharyngeal carcinoma were selected for LSC analysis. By histology 5 of the patients showed lymph node metastasis. By LSC all primaries had Cytokeratin^{+} populations. 1 metastasis and both histologically confirmed disease-free lymph nodes were Cytokeratin^{-}. None of the Cytokeratin^{+} cells in the primary and the metastases were diploid. The DNA index (DI) of the aneuploid peaks ranged from 0.8 to 3.9. %CV of the reference population of Cytokeratin^{-} cells was 4-6.5%. Both patients with disease-free lymph nodes were also aneuploidy. We found in 1 patient in the primary and in the metastasis an intensely green fluorescent population with DI=1.0. By re-localisation these cells were identified as eosinophils. As a control for Cytokeratin and DNA staining, pleomorphic adenoma of the parotid was used: This showed clearly epithelial, Cytokeratin^{+} cells with a DI of 1.0.

Conclusion: FNABs analysis by LSC yields reliable and objective data on the ploidy of malignant cells. The re-localisation feature allows to rule out any doubt about a given cell population. To make full use of the opportunities offered by the LSC protocols for multiparametric analyses have to be established.

4D-Imaging of Living Cells

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During the last few years we are gaining evidence that chromatin structure in particular, and nuclear organisation in general, is an essential element in the expression of genes. In the eukaryotic interphase nucleus, chromatin is spatially organized according to a number of slowly emerging principles. During mitosis the chromatin structure changes dramatically from a 3D structure (interphase nucleus) to essentially a linear organisation (metaphase chromosomes) and back to 3D (interphase nucleus of daughter cells). Amazingly, although chromatin is extremely dynamic during mitosis, its organisation in the two daughter cells is essentially the same as chromatin organisation in the mother cell and is apparently dictated mainly by the differentiation state of the cell. It is our goal to unravel the molecular mechanisms behind the redistribution of chromatin in the daughter cell nucleus.

In order to monitor chromatin movements in the newborn cell nucleus, we use HeLa cells with fluorescently labeled chromatin (expressing histone H2B-GFP), in combination with life cell confocal microscopy. Series of 3D-images of a single living cell are recorded as it proceeds from mitosis into G1. These 4D-images (3D + time) reveal the dynamics of chromatin during the first few hours of the newborn cell.

Multiplexed Flow Cytometry: An Emerging Technology
Synergistic Multi-Analyte Profiling Using Suspension Array Technology (SAT)

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The unique ability of flow cytometry, to make quantitative, homogeneous, multiparameter measurements has led to the development of a new class of molecular analysis that uses microspheres as solid supports. A historical look at current and potential future implementation of this technology will be covered. There is an extensive range of applications: immunoassays, generic analysis, high throughput screening of combinatorial libraries, and mechanistic analysis of molecular assembly. The continued expansion of flow cytometry into these new areas will depend on the development of improved assay chemistries to enable sensitive and reproducible quantitative analysis (higher avidity MAbs) and the development of microsphere platforma to support parallel analyses (in a range of biomolecular systems). Instruments in the Future will allow rapid analysis of large array of samples in

kinetic and/or endpoint assay format A perspective will be provided about practical and theoretical aspects of microsphere-based analysis. As a prototype example, the application for secreted cytokine assay panels will be covered. Both conventional and dedicated instrumentation for multiplexing will be considered. New systems permit the simultaneous measurement of multiple analytes. The multiplexed assay system has the capacity to perform simultaneously, multiple yet discrete homogeneous assays from a single small sample.. The effective application of multi-lasers optical configuration will also be described.

Reaktionen inflammatorischer Blutzellen auf partikuläre Noxen

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Verschiedene epidemiologische und tierexperimentelle Studien weisen auf einen Zusammenhang zwischen chronischen Lungenerkrankungen und der PM10-Partikelfraktion (Partikeldurchmesser < 10µm) in der Atemluft hin. Ziel unserer Untersuchungen ist es, die Reaktion von repräsentativen Abwehrzellen (Neutrophile Granulozyten, Monozyten und Lymphozyten) auf partikuläre Noxen in unterschiedlichen physiologischen Ebenen im Durchflußzytometer zu analysieren. Dieser Ansatz bezieht die Signaltransduktion einer inflammatorischen Response (Erzeugung von Ca2+ - Transienten) und Endpunkte der Inflammation (Respiratory Burst bzw. Apoptose) ein. Als Teilchenarten werden feine und ultrafeine TiO2-, Russ-, Printex-, und Ni-Teilchen, sowie Staphylococcus aureus konzentra-tionsabhängig untersucht. Die zeitabhängigen Ca2+-Messungen erfolgen mit der Indo-1-AM-Ratio-Methode und die Freisetzung von reaktiven Sauerstoffverbindungen wird mit über DHR123-Fluoreszenz gemessen. Zur Erfassung der Apoptose wird das Annexin-V-Assay herangezogen.

Die Untersuchungen zeigen, dass Printex-Teilchen in den drei untersuchten Zellpopulationen einen schwachen apoptose-verstärkenden Effekt induzieren. Staphylococcen verursachen eine leicht erhöhte Apoptoserate nur in Monozyten und Neutrophilen. Ni-Partikel zeigen einen geringen apoptotischen Effekt in Monozyten und eine sehr deutliche Wirkung auf Nuetrophile. Feine TiO2-, ultrafeine TiO2- und Russ-Teilchen als chemisch inerte Partikel sind dagegen nicht apoptotisch wirksam.

Diese Ergebnisse lassen vermuten, dass der mechanische Stress, der von Partikeln auf Zellen augeübt wird, allein nicht aureicht, um in den untersuchten Zeitintervallen Apoptose auszulösen. Vielmehr bedarf es zusätzlicher Einflüsse, wie chemische Toxizität, um modulierend auf die Apoptose zu wirken.

Analyse der Art der Fortpflanzung in höheren Pflanzen mittels durchflusszytometrischer Untersuchung reifer Samen

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Die bei verschiedenen Pflanzenarten vorkommende apomiktische (asexuelle) Vermehrung ist von großer wirtschaftlicher Bedeutung, weil sie die Fixierung positiver Eigenschaften, wie zum Beispiel von Heterosiseffekten ermöglicht. Dies erklärt die enormen Anstrengungen, die in der ganzen Welt zur Erzeugung apomiktischer Kulturpflanzen unternommen werden. Die sichere Unterscheidung apomiktischer von sich sexuell vermehrenden Pflanzen und der verschiedenen Typen der Apomixis ist eine Voraussetzung zur Entwicklung und züchterischen Nutzung der Apomixis. Bisher erfolgte dieser Nachweis mit indirekten Methoden (Nachkommenschaftstests oder Auxin-Test) oder es wurden mit großem Aufwand Endosperm- und Embryogewebe der Samen in bestimmten Entwicklungsstadien isoliert und die Chromo-somenzahlen ermittelt. Durch die doppelte Befruchtung bei Angiospermen entstehen Samen mit zwei verschiedenen Gewebetypen (Embryo und Endosperm), die sich im DNA-Gehalt unterscheiden: Während sich der Embryo aus der befruchteten haploiden Eizelle bildet und einen relativen DNA-Gehalt von 2C hat, entsteht das Endosperm, ein Nährgewebe für den Embryo, aus zwei haploiden Kernen der sogenannten Zentralzelle und einem Spermakern. Das Endosperm hat somit einen relativen DNA-Gehalt von 3C. Im Falle der asexuellen Vermehrung entstehen unreduzierte Embryosäcke. Der Embryo entwickelt sich ohne Befruchtung aus der unreduzierten Eizelle, und das Endosperm entsteht ebenfalls autonom oder nach Befruchtung. Daraus resultieren unterschiedliche Ploidierelationen im Samen (2C+3C=sexuell, 2C+4C=autonom apomiktisch, 2C+5C=apomiktisch mit einfacher Befruchtung). Diese Unterschiede lassen sich in reifen Samen trotz der geringen Zahl von Endospermzellen mit Hilfe der Durchfluss-Zytometrie nachweisen und bilden eine einfache Methode zur Ermittlung des Reproduktionstyps.

S-Phase Centromere Pairing in Stimulated Human Peripheral

Blood Lymphocytes and Repair Activated Cells

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Fluorescence in situ hybridisation (FISH) with Alpha-satellite DNA probes was applied to study the pairing behaviour of homologous chromosome centromeres in interphase nuclei of stimulated human peripheral blood lymphocytes. For several chromosomes, 1 , 3, 8, 15, X and Y, the distances between homologous chromosome centromeres were interactively measured and the distributions of the distances as a fraction of nuclear diameter were determined in more than 6000 cell nuclei. The S-phase nuclei were identified by antibody labeling of bromodeoxyuridine (BrdU) incorporation sites. The results showed a preferential somatic pairing of centromeres in S-phase of interphase nuclei. Simultaneous homologous centromere pairing of different chromosomes could not be observed using two colour FISH for centromeres 8/15 and 1/3. Further, we found a tendency of simultaneous pairing of the centromere and p-arm telomere of chromosome 3 in G1/S-phase enriched nuclei. After induction of DNA repair activity by H2O2/L-histidine treatment which induces DNA single and double strand breaks, an increase of centromere pairing from a value of about 39% ("without") to a mean value of about 49% ("with") was found for chromosomes 1, 15 and X. From the results it can be concluded that proliferating lymphocytes reveal a highly dynamic behaviour of centromeres in the S-phase and that chromosome pairing might play an important role in forming a correct DNA matrix for replication and repair.

Monitoring the I*n Vivo* Differentiation of Antigen-Specific T - Cells Following DNA Vaccination

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Vaccination with DNA coding for the antigen of interest is a versatile tool to induce cellular and humoral immune responses. To optimize vaccination strategies and to analyze the modulation of the specific immune response by certain vectors or vector combinations we have established a transfer system for direct analysis of antigen-specific T cells *ex vivo* following vaccination with DNA or antigens in adjuvant. Purified naive OVA-TCR-transgenic T cells were labelled with CFDA and transferred into congenic Balb/c recipients. Following immunization reactive cells can be identified according to transgenic TCR expression and loss of CFDA fluorescence upon cell proliferation. These reactive cells were directly analysed for the expression of phenotypic markers or the production of cytokines and chemokines upon restimulation *in vitro*. This system will give direct information on the differentiation status and the effector function of specific T cells *in vivo* in response to different immunization protocols. Based on these direct read-out of antigen-specific cells it will be possible to modulate vaccination strategies to obtain optimized immune responses.

Viable but Non-Culturable Cells in Microbiology – a Growing Problem !?

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The ability of bacteria to grow under the constraint of laboratory conditions has been a limitation in microbiology ever since the development of culture techniques by Robert Koch. Because of that, Koch used microscopy and took photographs for direct single cell analysis. Ever since culture methods have become more sophisticated and lead people to take culturability for granted and made it synonymous with the definition of viability. The advances in cytometry and the development of fluorescent probes have lead to a shift in the

terminology towards metabolic activity thus made us more aware of what is in a sample compared to what is growing from a sample.

To reduce the confusion around the term 'viability' this talk aims to give an overview of methods and terminology for the characterisation of bacterial cell function. Apart from a brief historic background examples of multicolour fluorescence are given to demonstrate how staining patterns can be characterised by multi-colour correlation and single cell sorting. Examples of cell injury will be given to illustrate the resulting population heterogeneity and how

cytometry has been used to determine the contribution of intrinsic and extrinsic oxidative damage to the lack of recovery from cell injury.

The Hydrolysis Derivative of Glucoraphanin but not the Native Glucosinolate Induces Differential Effects on Growth Inhibition, Cell-Cycle Arrest, and Induction of Apoptosis in Human T-Cell Leukemia.

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Glucosinolates (GLs) are naturally occurring compounds found in brassicas plants, which are capable of inhibiting, retarding or reversing experimental multistage carcinogenesis. When the plant tissue is broken, GLs come into contact and are hydrolyzed by the endogenous enzyme myrosinase (Myr), releasing a complex variety of products including isothiocynates (ITCs). Many synthetic ITCs, as sulforaphane, exert versatile chemopreventive effects against tumors induced by chemical carcinogens in various animal organs, modulating enzymes required for the carcinogens' activation or detoxification and/or the induction of cell-cycle arrest and apoptosis in tumor cell lines. In order to reproduce more closely the circumstances of dietary contact with sulforaphane following consumption of brassicas and to expand our knowledge on the chemopreventive potential of ITCs, we studied the effects of the mixture

of the GL glucoraphanin (GRA) and Myr on cell growth, cell-cycle progression and apoptosis induction in human T-cell leukemia Jurkat cells, also assessing p53, Bcl-2 and Bax protein expression, using various flow cytometric techniques. Only in the presence of Myr, GRA caused G2/M-phase cell-cycle arrest and apoptosis in a strictly time- and dose-dependent manner. These findings indicate that the hydrolytic product of the GRA-Myr mixture, sulforaphane, but not GRA alone, may exert protective effects. The end result is potent inhibition of leukemic cell growth. We also found that sulforaphane decreases Bcl-2 expression, but seems not to influence p53- or Bax-expression. We propose that sulforaphane be tested as a potent chemopreventive and chemotherapeutic agent for possible clinical use against human leukemias.

The Relation of Polysomies to Deletions of Chromosome 9 and Chromosome 17 in Early Lesions of the Human Bladder

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Aim: Increasingly Fluorescence in situ hybridization (FISH) is used as an apt method to improve sensitivity of cytological diagnostics by detection of polysomies indicative for aneuploidy, and to detect deletions. To validate the method it has to be defined how early and in which lesion genetic alteration arise within the urothelium.

Material and Methods: The urothelium of 41 early lesions (normal urothelium, hyperplasia, and displasia II) of a total of 36 patients was analysed according to centromeric distributions of chromosome 3, 7, 9 and 17 (probes: Vysis, Oncor) and a deletion of chromosome 17p13 (p53 Locus) as well as deletions of chromosome 9p21 (p16) and 9q22 (FACC). For all specimen a 2-colour FISH was carried out after microdissection to separate urothelium from stromal cells, and consequent nuclear preparation of the urothelium. As a control for hypridization efficiency a diploid urothelial cell line (UROtsa) was used. Polysomy was defined as an increase in chromosome numbers exceeding the mean of a control + 2 standard deviation.

Results: Inspite of microdissection and despite of small lesions an astounding heterogeneity was seen in terms of polysomy and detection of deletion. In contrast to dysplasias where polysomies are found regularly, hyperplasias rarely show polysomies of chromosomes. Polysomies of chromosome 9 and 17 are more frequent than those of chromosome 3 and 7. Nuclear correlation between chromosome 9 deletion and polysomy is found, however looking at deleted cells trisomy is found more frequently than disomy in those cells.

Conclusions: For screening of urine samples chromosome 3 and 7 are more likely to indicate aneuploidization than chromosome 9 and 17. The causal relation between chromosome 9 and p53 deletion and existence of polysomy has to be analysed by in-vitro studies in cell lines.

The MAINTRAC-Method Combining Magnetic Bead Enrichment and Laser Scanning

Cytometry (LSC) for Quantitation of Residual Tumor Cells in Blood and Bone Marrow.

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Purpose: Lymphomas and solid tumours are not only localisedevents but have systemic components which may be the cause of metastatic disease. Therefore it is important to know to which extent the tumours seed into the circulation. Ultrasensitive methods are necessary since, at best, 1 in 106 cells is expected to be detectable in bone marrow and perhaps even less in peripheral blood. PCR meets these requirements but requires a specific molecular marker.

Automated screening may help to search for cells which carry antigens present on the tumour cells but not in haematopoietic tissue. Methods: As a model system, mimicking seeding of tumour cells into the peripheral blood, clles of a tumor cell line (SKBr3) or FITC stained beads were diluted into normal peripheral blood to concentrations down to 10 cells or beads in 20 ml of peripheral blood. After red blood cell lysis cells or beads were labeled with magnetic bead antibody and enriched over magnetic columns. Enriched cells were applied to slides and screened by Laser Scanning Cytometry (LSC) for positive events. Up to 5x 104 cells were measured in suitable time and rescreened for morphological verification. Results: We were able to show that 1 positive bead was unequivocally detectable in 104 cells without previous enrichment and it was possible to re-liably recover 5-7 out of 10 beads/cells added to a 20ml blood volume after magnetic bead enrichment. Almost no beads/cells were lost due to washing or density gradients. Samples from 200 breast cancer patients and 50 controls have been measured and a reduction in circulating suspect cells upon therapy could be shown. Conclusions: This method is applicable for rapid and quantitative screening of blood for disseminated tumour cells for which specific surface or intracellular markers are available.It can be performed in a suitable time scale, making it possible for

the first time to correlate the number of cells detected with response to adjuvant therapy, metastasising properties of the tumour and prognosis. Additional morphological restaining, FISH analysis or in situ PCR is possible.

Microscopic Calcium Management in Cardiac Myocytes and Numeric Oscillator Simulations:
Insights into Pacemaker Development

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The development of autonomous pacemakers in ischemic parts of the human ventricle is a key process in the generation of life threatening polymorphic tachycardia (VPT) and subsequent ventricular fibrillation (VF). Herein the onset of a self- stabilizing oscillation in an oligocellular region and the spread of oscillation into the environment are critical steps. Nevertheless they are rarely explored.

By recording of the cytosolic calcium time course in a group of diffusion coupled ventriculocytes and the UV-microbeam based release of cytosolic calcium ions ("*Calcium Management*") it is possible to induce and to simultaneously observe the key steps mentioned above.

The UV- photolysis of Ca2+ from np-EGTA induces primary subcellular calcium homeostasis and calcium diffusion in parallel. The diffusion of calcium ions induces either secondary homeostasis (and subsequent oscillations) in the non- irradiated environment or it sensibilizes the environment to perceive the oscillator induction from the primary oscillator.

The extension of Fick's laws of diffusion with the non- linearly calcium dependent behavior of cytosolic calcium regulation, as known from electrophysiologically measured channel properties, gives a partial differential equation of the reaction diffusion type. This formulation is able to describe the experimentally measured reaction of ventricular tissue following to different initial calcium distributions.

The comparison of the numerically simulated oscillator with the microscopic results of the calcium management experiments allows to identify the characteristic properties of the cardiac tissue oscillator.

A Rapid and Sensitive Method for the Quantitation of Reverse Transcriptase-PCR Products Using Flow Cytometry

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Steady-state transcript level provides a global readout of the physiological state of a cell or a tissue. Alteration of specific gene expression often correlates with developmental changes or the cellular response to external components like hormones, viral infection, toxic compounds or irradiation. Therefore, the detection of changes of transcriptional activities can give important insights into gene and protein function as well as in the molecular processes of diseased cells. We developed a rapid and highly sensitive flow cytometric-based method for the quantitation of RT-PCR products. In this assay we used anti-digoxygenin magnetic particles to capture digoxygenin- and biotinylated-labelled amplification products and stained them simultaniously in a single step with streptavidin-R-Phycoerythrin. After a single washing step, the amount of fluorescent signals per bead was measured using a flow cytometer. We consistently detect PCR products down to 0.6 fmol. Additionally, this technique was used to determine the expression level of the p21/WAF1 gene, the proliferating cell nuclear antigen (PCNA) gene and the ß-actin gene in UVB exposed human keratinocytes. We conclude that this bead based assay is a reliable method for the determination of mRNA levels within 30 min and is appropiate for high throughput analyses.

Multiplex DNA- and RNA-Analysis Using Fluorescent Microbeads as an Alternative to DNA-Arrays

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Conventional DNA- or oligonucleotide-arrays consist of variable numbers of spatially separated nucleic acid spots. They may be used for sequence verification, expression profiling and other purposes. However, production of customized arrays is expensive, and for many clinical diagnostic purposes there is no need for high density arrays.

An interesting alternative are "suspension arrays" which represent a spectrally separated range of microbeads which can be identified by flow cytometry (Luminex technology). These beads may serve as a matrix for hybridization assays as well as immunoassays and others. Using four colour flow cytometry up to approx. 600-800 separate bead populations can be discriminated and used for hybridization assays. This number of specifications compares with intermediate density solid phase arrays and will be sufficient for most diagnostic purposes.

We have established and validated the Luminex technology in our laboratory for gene expression monitoring and mutation detection. Oligonucleotides specific for wild type and mutant sites in genes of interest are being coupled to differently stained beads and are used in competitive hybridization assays with PCR-products to identify the mutation of interest in a multiplex fashion. Multiplex-assays have been set up for detection of mutations in aa 609, 611 and 620 in the RET-protooncogene which is responsible for multiple endocrine neoplasia types 2A and 2B (MEN 2A, MEN 2B) and familial medullary thyroid carcinoma (FMTC). In addition, an assay for mutation detection in the newly identified ABCA1 gene responsible for familial HDL-deficiency syndromes has been established. Currently we are extending the possibilities of the Luminex technology using alternative detection technologies like hydrolysis probes, molecular beacons and minisequencing reactions and PCR on the beads.

Application of Laser Scanning Cytometry (LSC) in Toxicological Testing

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One of the main concepts in toxicology and risk assessment is the identification of compounds with the least toxicity, gaining increased understanding of the underlying mechanisms of efficacy and toxicity so as to accelerate the early selection of compounds for development. For that purpose, cutting-edge technologies, such as flow cytometry or confocal microscopy, have proved to be very valuable tools.

In our laboratory, we used laser scanning cytometry (LSC, CompuCyte Corporation Cambridge, Mass.) for cell proliferation, apoptosis and cell cycle analysis on skin and tumor samples from different species. This microscope-based cytofluorometer, which has attributes of both flow and image cytometry, quantifies the fluorescence of individual cells in cross sections or cytologis slides. It presents the advantages of rapidity and objectivity, in comparison with traditional immunohistopathological evaluation and is particularly well suited for the analysis of small specimens, such as those obtained from toxicological studies The possibility of combining these measurements with the visual image of the corresponding cell represents an advantage since morphological

structure can be preserved.

In conclusion, LSC has shown to offer a range of novel applications in areas within toxicology.

Laser-Scanning-Cytometry: A New Technique for Quantitative Analysis in Molecular Cytogenetics

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Laser-Scanning-Cytometry (LSC) as a diagnostic research tool automatically measures laser-exited fluorescence at multiple wavelenghts (blue light up to infrared with 4 photomultipliers) on slides featuring relocation of every single cell. Sperm were spread and fluorescence-labeled directly on the slide. In our first study particular chromosomes were tagged with fluorochromes being attached on centromeric or locus spezific DNA-probes whereas the chromatin was counterstained with DAPI. Missegregation of chromosomes during meiosis causes aneuploidy in sperm cells which may lead to severe syndromes in humans after fertilization. For comparing frequencies of spontaneous aneuploidy rates, FISH signals in epididymal sperm of young adult mice and human sperm were manually evaluated by fluorescence microscopy and automatically detected by LSC. Aneuploidy studies in germ cells require a precise knowledge of the duration of the meiotic stages. We applied LSC in a second study to measure the effect of the chemicals colchicine, diazepam, griseofulvin and vinblastine on the duration of meiosis in male mice. With thymidine analogues, such as bromodesoxyuridine (BrdU) it is possible to label cells at S-phase during preleptotene of meiosis. During meiosis I and II, 13 days later, the mice were treated with the test chemicals. In a time frame of 20-25 days after treatment, BrdU containing sperm were identified with a fluorescence-labeled anti-BrdU antibody. After colchicine treatment, the peak of BrdU labeled epididymal sperm occured significantly later than in the control group (day 24 vs. day 23, $p>0.01$). These data indicate that colchicine prolonged the duration of the meiotic divisions in mouse spermatocytes. The other tested chemicals showed no significant effect on meiotic cell cycle progression. It can be concluded that the quantitation of sperm with fluorescent signals by LSC saves time and is more objective than manual analysis.

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Laser Scanning Cytometry: A New Diagnostic Tool in Routine Cytology

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Laser-Scanning-Cytometry (LSC) is a newly developed technology to measure certain cytometric parameters like DNA-content and antigen expression or FISH. The method eliminates many of the drawbacks of flow-cytometry and image analysis.

We are using the instrument in routine diagnostic pathology for ploidy analysis of solid tumors and cytological preparations and can report on the results of ploidy measurements of over one hundred solid tumors. With the instrument we are also able to quantify immunological parameters e.g. hormon receptors or MIB1. It even is possible to calculate which fraction of the tumor is more or less positive. By means of multiparameter analysis immunotyping of lymphoma becomes also possible.

Patchwork-Images as a new Technique

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Teleconsultation (TC) offers an attractive possibility for exchange of ideas for pathologists. Mainly one problem causes dissatisfaction: The transmission of some prefabricated pictures is not conform to the attitude of a pathologist, to select coordinates and magnification by himself. Our Patchwork-Method tries to solve this problem by creating numerous images with the help of a motorised slide-tray, which scans a bigger part of the slide with a high magnification. All these pictures fit together to a virtual image, the Patchwork-Image. This is done by pathologist 1 (P1). After scanning the slide all the patchwork-images are transformed into one single overview-picture with normal size, which is compressed into a JPG-picture. P1 opens a session with pathologist 2 (P2), who, for example, is called for help in a diagnostic problem. The session begins with the transmission of the overview-JPG-picture. P2 then may select a region of interest in the overview picture, with a selected magnification. These 3 data (X, Y on the slide, magnification) are transferred to P1, and the software generates a new JPG-picture with the requested coordinates and magnification. The resulting picture will be transferred back to P2. P2 now can select a new magnification and a new coordinate from the resulting picture or from the overview-picture. This method has some advantages compared to remote-controlled microscopes : there is no need of a motorised objective-revolver, motorisation of focus also is not necessary. With known remote microscope

telepathology systems, one can discuss only with partners using the same system. Patchwork technology can be used by everyone who is connected to the internet and who uses a motorised slide tray. In our experience and in comparison to remote-controlled microscopes, this method is much more easy to use, the session works faster and helps to build up telepathology networks.

Biochemical and Cytological Analysis of Centromere Replication During the Cell Cycle.

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Centromeres are unique chromosomal loci that specify chromosome segregation in mitosis and meiosis and contribute to the spatial organization of the interphase nucleus. We have focused on a unique form of chromatin found in the kinetochore domain of human centromeres that contains a centromere-specific histone H3-related protein, CENP-A. CENP-A forms a modified nucleosome particle in alpha satellite DNA containing two copies of CENP-A with equimolar amounts of normal histones H4, H2A and H2B. Analysis of the replication timing of CENP-A associated DNA demonstrates that it replicates in mid-to-late S-phase, while CENP-A protein is synthesized later, in the G2 phase of the cell cycle. As histone synthesis is generally very tightly associated with DNA replication, the uncoupling of CENP-A synthesis from DNA replication suggests that regulated chromatin assembly plays an important role in kinetochore duplication. During G2, histone H3 is phosphorylated at serine 10 in order to promote chromosome condensation. Phosphorylation initiates in pericentric heterochromatin, where it goes to or near completion prior to the onset of chromosome arm phosphorylation. CENP-A possesses a serine residue in a conserved sequence context relative to histone H3 serine 10. Phosphorylation-specific rabbit antibodies were raised against a CENP-A phosphopeptide. Immunofluorescence demonstrates that CENP-A is phosphorylated in prophase, after histone H3 phosphorylation has gone to or near completion. CENP-A is rapidly dephosphorylated after the metaphase-anaphase transition. The kinetic separation of phosphorylation events, coupled with morphological duplication and maturation of the centromere provides an improved timeline with which to judge G2-M progression by cytological and cytometric methods.

Time-Resolved Fluorescence Measurements by Flow Cytometry

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A novel technology has been developed that combines fluorescence spectroscopy principles and flow cytometry (FCM) to provide unique capabilities for making time-resolved measurements in the frequency-domain on particles/cells labeled with fluorescent probes. No other instrument can quantify fluorescence lifetimes on a cell-by-cell basis in real time and resolve heterogeneous fluorescence based on lifetime differences, while maintaining the capability to make conventional FCM measurements. Cells are analyzed as they pass across a high-frequency, intensity-modulated (sine-wave) laser beam. Fluorescence signals are processed by 1) low-pass filtering to obtain conventional FCM measurements and 2) phase-sensitive detection to quantify lifetimes, including free fluorophore (solution), and resolve heterogeneous fluorescence. Processed signals are displayed as frequency distribution histograms and bivariate contour diagrams. Lifetime histograms recorded on autofluorescent cells; cells labeled with antibodies conjugated to fluorophores; cells, nuclei, and chromosomes stained with DNA-binding fluorochromes; and free and particle-bound fluorophore will be presented. Phase-resolved measurements will demonstrate the elimination of background autofluorescence in immunofluorescence studies and the resolution of signals from cells labeled with fluorochromes having overlapping emission spectra. This technology will increase the number of fluorescent markers usable in multilabeling studies and lifetimes can be used as spectroscopic probes to study the interaction of markers with their targets, each other, and the surrounding microenvironment.

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Measurement of Leukocyte Migration During Paediatric Cardiac Surgery by Flow and Laser Scanning Cytometry

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Cardiac surgery with cardiopulmonary bypass (CPB) induces massive perturbation of the immune system altering leukocyte composition in the peripheral blood (PB). This immune response contributes to the sometimes adverse outcome with capillary leakage and migration of activated cells to sites of inflammation that is driven by attractant and repellent chemokines acting in concert. In order to determine the chemotactic activity of patients serum obtained during and after surgery we established a chemotaxis assay for PB leukocytes (PBL). PBL from healthy donors were isolated (Ficoll-Hypaque centrifugation) and 250,000 cells were placed into a migration chamber (Costar) separated from a second lower chamber filled with patient serum (20% in RPMI) by a filter (pore width 3µm). After incubation (1h, 5% CO2, 37°C) cells from top and bottom chamber were removed and stained with a cocktail of 7 monoclonal antibodies for leuko- and lymphocyte subsets and analysed on a dual Laser FCM. Use of a cocktail was necessary as only a low volume of serum was available from the children. From both chambers the total number of cells recovered was 5-15% below that of the initial cell number due to attachment of migrating cells to the pores of the filter. These cells belong to the migrating compartment and were quantified by LSC after staining of nucleated cells by propidium Iodide (PI) and the whole filter was analysed (trigger=PI). Increased chemotactic activity started at onset of anaesthesia followed by a phase of low activity immediately after surgery and a second phase of high activity at post-operative days 1-2. In the first phase mainly monocytes and NK-cells migrated. The in vitro results correlated with results obtained by immunophenotyping of circulating PBL of the same patients showing that at CPB onset monocyte and NK-cell count increases. After surgery of T- and B-cell count decreased probably due to homing into lymphatic tissues. During paediatric cardiac surgery the chemotactic activity of the serum changes following characteristic patterns. Manipulation of the chemokine pattern might prove beneficial to prevent extravasation of cells leading to tissue damage. In chemotaxis assays with low amount of available serum and blood the combined use of FCM and LSC proved as a useful tool for analysis. (Support: Deutsche Herzstiftung, SMWK).

Magnetic Fields (MF) Influence Calcium Influx and Inhibit the Extent of Apoptosis Induced by Chemical and Physical Agents in Human Glioblstoma Cells

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Epidemiological data registered a recent increase of brain tumors, rising concerns about the possible link with MF exposure. It is not clear how MF are transduced into biological signals to produce toxic outcome on brain cells. Evidences indicated that the effects are mediated by Ca^{2+} influx.

The effect of exposure of a human glioblastoma primary culture to 6 mT MF on the intracellular Ca^{2+} was investigated by dual-emission microfluorimetry. Being Ca^{2+} ions crucial messengers in the apoptotic commitment, the interference of MF on the apototic induction by chemical and physical agents was also investigated.

The exposure of the cells to 6 mT MF triggered an increase of Ca^{2+} from basal values of 128 $\pm$ 9 nM to 290 $\pm$ 55 nM ($p < 0.01$). The increase was likely due to the capacitative Ca^{2+} entry . In fact, cytosolic Ca^{2+} was uneffected by MF when extracellular Ca^{2+} was blocked by chelation with EGTA. The rise of Ca^{2+} showed a slow kinetic and long-lasting high cytosolic values compared to the rise of Ca^{2+} due to the addition of 10 nM extracellular Ca^{2+}. The influence of MF as an apoptogenic stimulus was negligible. Conversely, the presence of 6 mT MF dramatically reduced the extent of apotosis induced by either chemical (VP 16) or physical (42,5° C) agents, being the rescue of 46% and 82% respectively. These results are confirmed by both microscopic and flow cytometric analysis. Thus, MF increase cytosolic Ca^{2+} and reduce the extent of apoptosis induced by Vp 16 and hyperthermia in glioblastoma cells, allowing the survival of possibly mutated cells.

Expression von CD64 durch neutrophile Granulozyten bei inflammatorischen Prozessen des Gastrointestinaltraktes

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Hintergrund:

Neutrophile Granulozyten (PMN)sind wichtige Effektorzellen bei inflamma-torischen Prozessen. Der hoch-affine Rezeptor für Imunglobulin G (CD64) wird von PMN bei Gesunden praktisch nicht exprimiert. Funktionell ist die Expression von CD64 assoziiert mit erhöhter ADCC und Aktivierung des NADPH-Oxidase-Systems. Bei Patienten mit chronisch-entzündlichen Darmerkrankungen (CED) wurde eine deutliche up-regulation beobachtet.

Fragestellung:

Es wurde die CD64-Expression durch PMN bei Patienten mit akuter Pankreatitis und infektiöser Enteritis untersucht. Als Vergleichskollektive dienten Gesunde, Patienten mit CED und Patienten mit Sepsis.

Methodik:

Die Messung von Cd64 erfolgte aus dem Vollblut mittels FACS-Analyse. Es wurden jeweils FITC-markierte Antikörper verschiederner Klone eingesetzt (Immunotech 22, Pharmingen 10.1, Medarex 32.2), deren Vergleich eine enge Korrelation zeigte. Die statistische Analyse erfolgte mittels t-Test (Patienten vs. Gesunde).

Ergebnisse:

Die Ergebnisse sind als Median/Interquartilabstand des Prozentsatzes positiver PMN angegeben. Die höchste CD64-Expression fand sich bei Patienten mit Sepsis (n=13, 99/13%, p<<<0,0001). Patienten mit akuter ankreatitis (n=36, 73/51%, p=0,0001) und infektiöser Enteritis (n=14, 93/57%, p<0,0005) zeigten ähnliche Werte wie Patienten mit aktivem M. Crohn (n=23, 64/47%, p<<0,0001)und aktiver Colitis ulcerosa (n=11, 82/37%, p<<0,0001). Bei Patienten mit M. Crohn in Remission (n=16, 34/36%, p=0,05), nicht jedoch mit Colitis ulcerosa in Remission (n=9, 21/74, p=0,18) waren die Werte gegenüber Gesunden (n=10, 22/13) signifikant erhöht.

Diskussion:

Patienten mit akuter Pankreatitis und infektiöser Enteritis weisen eine ähnlich hohe Expression von CD64 auf wie Patienten mit aktiver CED. Dies unterstreicht die pathogenetische Bedeutung der PMN-Aktivierung bei diesen Krankheitsbildern. Klinische Bedeutung könnte CD64 für die Differentialdiagnose chronischer Durchfallserkrankungen erlangen. Hinsichtlich der Bedeutung von CD64 als Akut-Phase-Parameter sind weitere Studien erforderlich.

Anomalos Diffusion Inside Living Cell Nuclei Investigated by Fluorescence Correlation Spectroscopy

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We have investigated spatial variations of the diffusion behaviour of fluorescent markers in living cells with fluorescence correlation spectroscopy (FCS). Our FCS device provides submicron spatial resolution and a detection volume smaller than a femtoliter. The diffusional contribution to fluorescence fluctuations deviates from ideal behaviour and depends on the position in the cell. The data can be evaluated as two components undergoing free Brownian motion. Besides a fast component with a diffusion coefficient approx. 5 times smaller than in aqueous solution a slow component appears especially in the nuclei diffusing one or two orders of magnitude slower. Alternatively we can fit the data to an anomalous diffusion model where the degree of obstruction of the free motion is large again especially in the nuclei. Possible mechanisms for this long tail behaviour include corralling, immobile obstacles, and binding with a broad distribution of binding affinities.

We have added a sample scanning and a laser scanning device to our instrument which now provides "imaging functionality". With this setup, we can characterize the distribution of the structures that are expected to bias the diffusion properties, *e.g.* in nuclei of cells expressing histone-GFP-fusion proteins, and simultaneous recording of FCS data of spectrally distinct fluorescent markers at defined positions provides results that can be compared with recent numerical models of the chromosome territory structure in the cell nucleus. Especially geometrical properties of the interphase chromatin are expected to be strongly correlated to the degree of obstruction of the diffusion behaviour.

Detection and Quantification of PCR Products Using Flow Cytometry

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Quantification of PCR products is of great importance for the detection of gene expression changes, of pathological genome alterations, and of infectious germs. However, sensitive assays to quantify amplification products are often

too laborious or expensive.

Here we describe a flow cytometry-based assay for a sensitive quantification of PCR products in less than 25 minutes. In this assay PCR products are both digoxigenin- and biotin-labeled during amplification. Subsequently, amplicons are simultaneously bound to microparticles coated with anti-digoxigenin antibodies and fluorescently labeled using streptavidin-R-Phycoerythrin. Finally, the average fluorescence intensity per bead is determined by flow cytometry. This quick assay is of high reliability and allows the detection of PCR products down to 0.4 fmol, corresponding to e.g. 40 pg of a 163 bp amplification product. These attributes predestinate our assay for various PCR applications.

To demonstrate the potential of this assay we quantified the expression levels of various genes following UV-B- and X-irradiation of human keratinocytes and leucocytes, respectively. As an example for a clinical routine diagnosis cytomegalovirus was detected in whole blood samples. In addition we show that the flow cytometric assay also allows the quantification multiplex PCR products using two populations of microbeads with different binding specificities simultaneously.

The Process of Replication in the Context of Stably Positioned Chromatin

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Recently we demonstrated that chromatin with defined functional features is organized into specifc higher order compartments within mammalian cell nuclei. Higher order compartments are established after mitosis and are maintained during whole interphase. Our data from live cell microscopy studies of single human chromosomes or chromosomal subcompartments support the idea that chromatin is mainly stably positioned. The question emerges how dynamic processes like DNA replication can take place within stably positioned chromatin. In particular the widely accepted idea that DNA is spooled through fixed replication factories implies that chromatin should be highly dynamic at least during S-phase. In order to investigate the dynamics of the replication process we labeled replication factories with a PCNA-GFP fusion protein. Confocal time lapse series of living cells revealed that also replication factories are positionally stable. However, there is a constant and asynchronous process of assembly and disassembly of replication factories.

As a whole the data suggest the following scenario: chromatin with a specific replication timing is organized in G1 into specific higher order compartments. During S-phase replication factories assemble at the appropriate stage of S-phase within the compartment that is replicated at this specific stage. After local chromatin replication the factories disassemble. Currently we are investigating the local interactions between chromatin and replication factories.

Poster presentations (alphabetical order)

Cytophotometric Study of Cardiomyocyte Ploidy Level in Birds with Different Growth Rate and Lifestyle

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Somatic polyploidy, or multiplication of genome number is a widespread phenomenon. Polyploid cells were discovered in all mammalian tissues but nothing is known about other vertebrates, birds including. In present study the ploidy levels in ventricular cardiomyocytes of 36 adult bird species, belonging to 9 orders, were estimated using smears of isolated cells stained by means of the Schiff-Auramin-SO2 technique. It was found that the 2cx2 myocytes were predominant in the adult bird myocardium where they accounted for at least 50% of the cell population. Multinuclearcells with 3 to 8 diploid nuclei were widely spread. The percentage of such cells was 5-6 times higher in precocial than in altricial birds of the same weight. The myocytes with polyploid nuclei were rare. A large interspecies variability of cardiomyocyte ploidy levels was found. The most prominent differences were revealed between the precocial and the altricial birds. At the same time, the mean number of genomes in cells was correlated both with the body mass and the growth rate of birds. The differences between the precocial and altricial birds disappeared on elimination

(with the aid of a statistical method) of the effect of growth rate but did not when the effect of body mass was

eliminated. Among the altricial birds, which are basically immobile during their growth, the cardiomyocyte ploidy levels also

correlated more closely with the growth rate than with the body mass. The opposite was observed in the precocial birds which are highly mobile from the first minutes of life. It is concluded that the interspecies variability of the bird cardiomyocyte ploidy levels is a result of shift in the balance between the cardiac functional load and the growth rate, which is manifested at the cellular level as a competition between the proliferation and differentiation of cardiomyocytes.

Activity of Glucose-6-Phosphatase in the Hepatic Lobule Zones of the Cirrhotic Rat Liver

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Activity of glucose-6-phosphatase (G6Phase) was studied by a histochemical method in the portal and central zones of the rat liver in norm and experimental cirrhosis. Experimental liver cirrhosis in the rats was produced by a 6-month-long inhalation of carbon tetrachloride (CC14). G6Phase was detected by prefixation of cryostat sections in a fixator with addition of glutaraldehyde and by a subsequent prolonged (18-24 hr) ncubation of the sections in a chilled standard medium. To vizualize the reaction final product, precipitate of lead phosphates, it was necessary to replace the anion by sulfide, which provided conversion of colorless products into the stained ones. Activity of G6Phase was determined using a Videotest image analyzer (Ista-Videotest, Ltd., St. Petersburg, Russia) that allowed combining cytospectrophotometric analysis of the content of the cell compound with determination of its precise localization in the tissues. It has been shown that in norm, activity of G6Phase in the portal zone was 1.5 times higher than in the central zone of the hepatic lobule. The ratio of the G6Phase activity in the periportal and pericentral (P/C) hepatocytes is, on average, 1.35. In rats with liver cirrhosis the G6Phase activity in the portal zone hepatocytes was decreased by 26%. The ratio P/C in the cirrhotically altered liver, the zonal heterogeneity of G6Phase is preserved, although it is changed significantly as compared with norm.

Enrichment for Late Telophase Cell Populations Using Flow Cytometry

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In modern flow cytometry the "pulse processing" is a commonly used tool to exclude cell aggregates from cell-cycle analysis which involves deconvolution of single-parameter data with a three-compartment model (G1, S, G2+M). However, in our view, exclusion of cell aggregates among which dividing cells of the final stage of mitosis, particularly late telophase cells, are presumed to be present may affect mitotic indices evaluated by flow cytometry. In order to test this presumption, HeLa S3 (human cervical carcinoma) cells were stained with propidium iodide (PI) for DNA, and the area of the PI fluorescence signal in combination with the signal height (FL2-A vs. FL2-H) was utilized to discriminate late telophase cells which might be present along with aggregates of two G1 cells prior to their sorting. Cells with morphology characteristic to late telophase cells were identified and quantified in the fraction of sorted cells. They were found to constitute no less than 7% of manually counted mitotic cells. Thus, the amount of late telophase cells discriminated by means of pulse shape analysis can be sufficient to alter the accuracy of mitotic indices scored in the flow.

Several flow cytometric methods have been proposed for quantitative evaluation of mitotic indices as a rapid alternatives for microscopic examination, and among these the methods based on immunocytochemical detection of mitotic cells seem to be most attractive (1-4). However, since no evidence has been shown that all cells by the end of cytokinesis, especially late telophase cells, can be identified or efficiently identified by mitotic marking antibodies, the accuracy of these methods remains to be a subject of critical comments. This might be associated with the fact that some molecular processes (e.g., changes in phosphorylation/conformation state of specific mitotic markers-proteins composing nuclear chromatin) accompanying reconstitution of daughter nuclei in the final stage of mitosis can reduce the binding ability of these antibodies. In this regard, late telophase cells enriched by "pulse processing" are believed to provide a clue to a more accurate evaluation of mitotic indices based on immunocytochemical staining.

(oral)

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Functional Apoptotic Responses of Jurkat T-Cells Analysed by Time-Resolved Flow Cytometry

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Programmed cell death is a main focus of interest in a variety of scientific and clinical areas. For analyzing very early apoptosis-related events, we have established a flow cytometric technique to quantify time-dependent signals in living cells with high temporal resolution of 1 s over 17 min. For this purpose, a thermostatted sample tube holder was developed for repeatable interruption-free injection of substances into the cell suspension. Apoptosis was induced

by the biozid Tri-n-butyltin (TBT) in Jurkat T-cells. Early detectable fluorescence and scatter parameters were related to intracellular free calcium concentration (Indo-1 fluorometry), membrane permeability (propidium iodide (PI) influx) and cell volume (forward scatter). These early indicators of a possible apoptotic outcome were correlated to the Annexin-V assay as an indicator of apoptosis. TBT-concentrations of 0.5 to 10 µM induced a rapid transient increase of intracellular free calcium only in a fraction of cells (Ca-TR); in an other subpopulation a steady state calcium signal (Ca-SST) was observed. Analysis of the simultaneously registered PI-signals showed a shift to increasing PI-fluorescence in Ca-SST cells only, accompanied by a TBT-concentration dependent decrease in cell volume during this early time period. For TBT concentrations between 1 and 5 µM the fraction of Ca-SST cells was found to be similar to the fraction of Annexin V - positive cells between 2 and 3

hours after induction of apoptosis. These findings indicate that, at least in our model system, very early functional changes in intracellular free calcium are well suited as prognostic assessment for a later apoptotic outcome.

Flow Cytometric Examinations on the Effect of New Antibiotics of the Leptomycin Family on Tumor Cells

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As flow cytometry is a rapid method to analyse the state/ condition of cell cultures it can be used for in vitro studies. In vitro studies on cell- or tissue cultures are well known in pharmaceutical research. It is the first step to determine the effect of a new substance on a living organism. Only after finishing these studies the active substance is tested on animals and finally on human.

Ratjadon is one of these new substances with promising effect against tumor cells. It belongs to the well examined Leptomycin family. All known Leptomycins (Leptomycin A, B, Kazusamycin A, B, Dilactonmycin, Delactonmycin) inhibit the regulatory gene Rev translocation at nanomolar concentrations.

We focused on the basic investigations, dose-effect connection relationship. The permanent T-lymphocyt cell line Jurkat and the permanent glioblastoma cell line U87-MG are used to measure the vitality and cell number at different ratjadon concentrations. Furtheron we examine the cell cycle phase at which ratjadon causes arrest. Also determinations of pH and membrane potential are carried out.

In the future several ratjadon derivatives will be examined towards their structure activity relationship.

Zonal Heterogeneity of the Glycogen Content in the Liver Lobule Zones of Patients with Liver Cirrhosis

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By cytophotometric method, the concentrations of total glycogen (TG) and of ist labile fraction (LF) and stable fraction (SF) were determined in hepatocytes of portal and central zones of the normal human liver and in the liver of patients with liver cirrhosis of viral and alcohol etiology. Using PAS reaction, TG and its LF and SF were revealed in histological sections of the material obtained by the liver punction biopsies. The concentrations of TG and its fractions were measured by televisional cytophotometry. In liver cirrhosis, the concentration of TG, LF, and SF in both zones of the hepatic lobule have been found to be much higher than in the normal liver. It has been shown that the ratio of the hepatocyte TG concentrations in the portal zone to the central zone (the P/C ratio) both in norm and in viral cirrhosis does not significantly differ from that in norm. On the contrary, in the liver of patients with alcoholic cirrhosis, the P/C ratio is reduced to 0.815+0.016 and is accompanied by qualitative changes of the glycogen composition. Thus, quantitative cytophotometric analysis of glycogen content in liver lobule zones may be the marker in the diagnosis of liver cirrhosis with different etiologies.

Differentiation of Bacteria Grown in Mixed Culture Using Lectin Binding

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The gram-positive bacterium Rhodococcus erythropolis K2-3 and the gram-negative Ochrobactrum anthropi K2-14 are capable of synergistically degrading 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB). The two strains execute this task in a symbiotic manner, but the nature of the interactions involved in the degradation is only partially understood as yet. An essential first step in elucidating the interaction is to be able to monitor the two strains separately, at the cellular level, within mixed populations. Therefore a method exploiting fluorescently labelled lectin probes was developed. Since Concanavalin A (Con A) binds specifically to R. erythropolis K2-3, it was selected and linked to the fluorescent dye Bodipy 630/650, with has an excitation maximum in the red part of visible light spectrum. Forward light scatter (FSC) and DNA fluorescence from both strains were also measured to obtain simultaneous information about their physiological states. The three parameters were conveniently monitored by dual and triple excitation flow cytometry in conjunction with double fluorescent staining techniques. In addition, the strains were identified using an epifluorescence microscope. These techniques were found powerful tools for the population analysis of this mixed bacterial system.

Rapid Fixation with Peroxyacetic Acid and Chlorine Dioxide Compared to the Fixation with Alcohol

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The analysis of the cell-physiological conditions of yeast, which is used during the production process in a brewery, is done by a flow cytometer. Current technology for sample fixation is the use of alcohol. However, this technique has the disadvantage that every sample pretreatment takes minimum three hours. Accordingly it is not possible to execute the analysis fast and flexible during the production process and to have the possibility to react. Therefore, a time-saving method was developed.

A new fixation technique with peroxyacetic acid and chlorine dioxide solutions was evaluated followed by the analysis with a Coulter Epics XL – MCL flow cytometer. The measured parameters for the determination of the physiological conditions are nonpolar lipids, glycogen, DNA and live-dead determination. The result have been compared with the live-dead-determination of the yeast cells by a fluorescence microscope method.

The fixation with peroxyacetic acid and chlorine dioxide is a good alternative to the classical fixation with alcohol. Both fixation techniques could use the same cytometer parameters.

Assessment of Bacterial Viability Status by Flow Cytometry and Single Cell Sorting

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The fluorescent staining methods developed in our laboratory have lead to a functional classification based on

reproductive activity, metabolic activity and membrane integrity. The multicolour staining approach has improved the understanding about various dye properties (Nebe-von Caron,G. et al, 1995) and also highlighted the interference of dye efflux systems, a major cause of interference and misinterpretation of fluorescent stains. In case of those systems being proton antiport pumps (Midgley,M. 1986) their deliberate measurement can be used to discriminate for cells still generating a proton gradient. Combination with membrane potential and membrane integrity staining allows simultaneous differentiation of four functional sub-populations in naturally heterogeneous populations. The reproductive growth capacity of intact cells can be demonstrated by cell sorting (Nebe-von Caron,G et al, 1998).

With heat injured cells different media are known to show up to 4 log of differences in recovery (Stephens,P. et al 1997) already contributing to the legacy of viable but non culturable cells (VBNC's). Cytometric cell sorting demonstrated decreasing recovery with increasing loss of membrane function. Comparison of a 'good' performing with a newly developed pre-enrichment medium that protected the cells from intracellular and extracellular causes of oxidative stress showed increasing benefit of oxidative protection with prolonged injury and improved recovery considerably. Active respiring cells showed much higher recovery improvement than the other populations, demonstrating for the first time the contribution of active respiration to intracellular cause of oxidative damage as key part of the VBNC problem. This highlights the advantage of measuring cell function at the single cell level and raises questions regarding safety of classical viability assessment.

Durchflußzytometrische Bestimmung der Chinolonempfindlichkeit von Streptococcus Pneumoniae und Staphylococcus Aureus

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Die durchflußzytometrische Bestimmung der Antibiotikaempfindlichkeit von Bakterien mit Hilfe membranpotentialsensitiver Farbstoffe ermöglicht das Austesten und den Vergleich antibiotisch wirksamer Substanzen.

Untersucht wurde die Wirkung der Chinolone Moxifloxacin, Levofloxacin und Ciprofloxacin auf das Membranpotential der grampositiven Bakterienstämme Staphylococcus aureus und Streptococcus pneumoniae. Chinolone hemmen die bakteriellen Enzyme Gyrase und Topoisomerase IV, die zur Nukleinsäure-Synthese benötigt werden und stören so die Zellteilung. Bei Schädigung der Bakterienzelle durch das Antibiotikum kommt es zur Depolarisation der Zellmembran, der Farbstoff $DiBAC_4(3)$ gelangt ins Zellinnere und die Bakterien fluoreszieren grün.

Von 50 Stämmen Staphylococcus aureus und 40 Stämmen Streptococcus pneumoniae wurden die Chinolonkonzentrationen bestimmt, ab denen eine nachweisbare Membranschädigung der Bakterienzelle erfolgt.

Die Ergebnisse wurden mit dem Agardiffusionstest und mit Antibiogrammen des WalkAway96Ò bzw. des Vitek II verglichen.

Sowohl bei Staphylococcus aureus, als auch bei Streptococcus pneumoniae zeigte sich, daß Moxifloxacin bereits in geringeren Konzentrationen zur Depolarisation der Bakterienmembran führt als Levofloxacin oder Ciprofloxacin.

Ciprofloxacin zeigte im Vergleich mit Levofloxacin bei Staphylococcus aureus eine etwas geringere Wirksamkeit.

Alle Bakterienstämme konnten mit den anderen Verfahren übereinstimmend als sensibel oder resistent eingeordnet werden.

Snom of Breast Cancer Cell Surfaces after Structure Conserving HMDS Supported Air Drying

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Scanning near-field optical microscopy (SNOM) can simultaneously map topographic and optical properties with a

spatial resolution in the 100 nm range. SNOM for biological applications poses several challenges such as imaging of soft samples or large topographical changes. For principle reasons in the routinely applied shear-force feedback technique dry objects are required. Therefore an easy to handle specimen preparation protocol for cells was developed using a test system of fresh blood cells from mouse. A commercially available transmission SNOM (SNOM 210, Carl Zeiss Jena, Digital Instruments) with micro-fabricated silicon nitride tips mounted in a shear-force sensor head was used. The instrument was equipped with an argon ion laser (458 nm, 488 nm) and two HeNe lasers (543 nm, 633 nm). Absorption or fluores-cence could be detected by a photomultiplier or an avalanche photo diode. Based on a standard Zeiss Axiovert 135 microscope far-field imaging was also possible.

Breast cancer cells of the cell line T-47D grown on glass slides were pre-treated in a modified culture medium free from residual estrogens and components with similar activities. Aliquots of the cells were treated with estradiol of different concentrations for 48 hours. After fixation with formaldehyde the specimen was dehydrated by an ethanol series and air dried after hexamethyldisilazane (HMDS) exposure. HMDS is known to reduce surface tension and to cross-link proteins so that the shape of cell surfaces were conserved. Air drying without HMDS treatment destroyed cellular shape and surface morphology.

From far-field images regions of interest (typically about 2 µm x 2 µm) on certain cells were selected. The shape of cellular surfaces was then imaged by near-field scanning according to topography and absorption. Changes in the morphology were found after estradiol treatment in comparison to the untreated control which indicated a treatment response. These changes may be assigned to stimulation of proliferation and/or secretion. According to our knowledge these are the first systematic optical near-field investigations on cancer cells.

Cytophotometric Assessment of Protein Contents and the Number of Genomes in Hepatocytes of *Cryptosporidium Parvum* infected Suckling Rats

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Cryptosporidiosis is a widely spread intestinal disease of animals and humans caused by the intracellular parasitic protozoa of the genus *Cryptosporidium* (Sporozoa, Apicomplexa). The most characteristic feature of cryptosporidiosis is a waterty diarrhea, often resulting in body dehydration and muscle dystrophy. Up to now almost nothing has been known about the influence exerted by *Cryptosporidium* on the inner organs other than intestine. In the present study changes in the liver of *C.parvum-* infected suckling rats have been first investigated at the cellular level using methods of cytophotometry and fluorimetry. 12 day old rats were fed *C.panvum* oocysts in different doses - $3.5x10^5$ and $1X10^6$ to obtain, respectively, a weak and a heavy infections. The rats were sacrificed 4 days after infection. The relative liver weights in the infected rats of the respective groups exceeded those in the control by 15±0.8% and 32±1.7% At the cellular level the typical response of hepatocytes in pathologically changed liver is the augmentation of protein content and the ploidy levels in these. The protein contents in hepatocytes were estimated on smears of isolated cells using absorption cytophotometry after Naphthol Yellow staining. In the weakly and heavily infected rats the protein contents, reflecting the functional activity of hepatocytes, increased, respectively, by 10±2.3% and 30±1.7%. The ploidy of hepatocyte nuclei was measured fluorimetrically using the Schiff-Auramin-SO2 technique (Kasten, 1961). Again, in the weak and the heavy infections, respectively, the share of tetraploid hepatocytes (2c x 2 and 4c) was seen to increase by 25±1.2% and 100±6.3% .It is to be noted that in the latter case some octaploid cells (4c x 2 and 8) appeared, making near 1.7±0.2%, which are never observed in the normal group of rats. Thus, the data presented suggest that the infection of the rats with the intestinal protozoan pathogen, *C.parvum,* involves in addition some obvious pathological changes in the host liver, in spite of the fact that this parasite does not exert any direct influence on the liver itself, either mechanical or chemical.

Detection of Aromatase Protein in Bovine Lutein Cells by Flow Cytometry

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Introduction: Ovarian follicle cells differentiate into lutein cells to produce pregnancy-maintaining steroids (progestins). Progesterone production requires estradiol synthesized by P450 aromatase. However, P450 aromatase expression in bovine lutein cells is not known. The aim of the study was to examine expression of P450 aromatase in bovine lutein cells.

Methods: Lutein cells were obtained from corpus luteum on day 5 and 12 of the ovarian cycle. P450 aromatase protein was detected by imaging, flow cytometry, and immunoblotting. Steroidogenesis was measured using RIA and EIA for progesterone and estradiol.

Results and Conclusions: P450 aromatase was detectable by immunblotting, microscopic imaging and flow cytometry. The latter enables specific detection of P450 aromatase in small and large lutein cells. The large cells

(day 5) expressed an amount of the enzyme threefold the amount of the small cells. Decrease in the amount per cell occurred at day 12 in both cell types. Expression correlated with steroidogenesis. The results demonstrate that bovine lutein cells express P450 aromatase, the expression is dependent on the developmental stage of the corpus luteum, and estradiol-17 b production is correlated with expression rate of P450 aromatase.

Flow Cytometric Analysis of Cell Membrane Dependent Chromatin Condensation

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It was shown that after cell membrane damage chromatin undergoes rapid decondensation, at least part of which is due to the difference in solvent composition inside and outside the cell. This process can be monitored by means of flow cytometry with the use of cross-linking fixative agents and staining with DNA-specific fluorochromes. The ratio of fluorescence intensity of the intact to the membrane-damaged cells can be 2-4-fold. This approach provides a good model to study the action of different solvents on the chromatin condensation state, applied either within (after cell membrane being made permeable) or outside the cell. Also, this technique can be used to study the chromatin condensation state in different cell types. Especially valuable this approach can be for cell viability assessment. It has two advantages over the traditional methods where cell viability is estimated using live cells: 1) no cell death occurs during the measurement, and 2) the measurement can be postponed up to several days after the sample collection. Thus, it can be used, for instance, in the large-scale toxicological experiments where cell samples can be fixed rapidly in the real time and analyzed later. Furthermore, this method gives the possibility of monitoring cell dying process on the time-scale with an increased resolution (with a time-step below 1 min), which methods using measurements of live cells cannot furnish. Also it can be applied in the environmental studies where samples collected in the field can be analyzed afterwards in the laboratory.