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ABSTRACTS

1

CLARIFYING AMBIGUITIES IN ACUTE LEUKEMIAS OF AMBIGUOUS LINEAGE.

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Background: Understanding of clinico-pathologic and immunophenotypic-genotypic significance of acute leukemias with immunophenotypic aberrancies is limited due to lack of consensus in the literature in criteria used to define these entities. The World Health Organization (WHO) recommends a scoring system proposed by the European Group for the Immunologic Classification of Leukemia (EGIL) for markers used in lineage-associated assignment in acute leukemias. However, two key issues are not addressed by this scoring system: 1). Low intensity expressions; 2). Subpopulation expressions. **Method**: We retrospectively studied the pathologic significance of immunophenotypic aberrancies in 80 cases of acute leukemias using the scoring system recommended by the WHO, plus our newly proposed criteria that aberrant expressions were considered positive if either >5% of total blasts showed moderate to high intensity expression, or low intensity expression of >20% was detected. **Results**: We detected 7 cases (8.7%) of acute ambiguous leukemias (biphenotypic or bilineal), with t(9;22) in 4 out of the 7 cases. 36 cases (45%) were acute lymphoblastic leukemias with aberrant myeloid expressions including, most commonly, CD15, CD33, and CD33, 56% of which had multiple cytogenetic aberrations. The remaining 37 (46.3%) cases were acute myelogenous leukemias with aberrant lymphoid expressions including, most commonly, CD7, CD56, and CD2, 33% of which revealed multiple cytogenetic aberrations. Clinico-pathologic and immunophenotypic-genotypic correlations of the findings are being evaluated. **Conclusion**: The criteria for defining immunophenotypic aberrancies in acute leukemias remain unclear. We hope that our proposal can trigger interest in developing guidelines that would offer added sensitivity and specificity in identifying immunophenotypic aberrances of acute leukemias and their clinical significance.

2

DETECTION AND QUANTIFICATION OF CIRCULATING TUMOR CELLS IN MOUSE MODELS OF HUMAN BREAST CANCER USING THREE-COLOR FLOW CYTOMETRIC ANALYSIS.

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Circulating tumor cells (CTCs) in the peripheral blood of breast cancer patients can be detected by flow cytometric analysis, and may be an important indicator of metastatic disease and prognosis. Techniques to detect, quantify, and characterize rare cells in peripheral blood are imperfect. Similar challenges in detecting and quantifying rare metastatic cells in mouse models has hindered the ability to fully investigate and understand the kinetics of the metastatic process. The purpose of this study was to assess and optimize the sensitivity of flow cytometry for detection of human breast cancer cells in mouse blood samples. Set percentages of MDA-MB-468 human breast cancer cells (50-0.005%) were added to fresh samples of blood obtained from female athymic NCr nude mice via terminal cardiac puncture. Samples were incubated with a FITC conjugated anti-HLA antibody and a PE conjugated anti-mouse pan-leukocyte CD45 antibody, fixed, permeabilized, and stained with propidium iodide (PI). Human MDA-MB-468 breast cancer cells could be differentiated from mouse white blood cells based on their increased light scatter, positive staining with anti-human HLA-FITC, negative staining with anti-mouse CD45-PE, and aneuploid DNA content on PI staining. Criteria were also effective for detecting MDA-MB-231, MDA-MB-435, and 21NT human breast cancer cells in whole mouse blood. The lower detection limit for sensitivity was 0.01%, or 10-4. This detection sensitivity is equivalent to 1 tumor cell per 10,000 white blood cells. The sensitivity of the technique could be increased to 10-5 by using an immunomagnetic method for depletion of the CD45-positive white blood cells prior to flow cytometry analysis. This technique has the potential to be a valuable tool for determining the kinetics of early steps in metastasis and for investigating the biological relevance of CTCs in experimental mouse models of metastatic breast cancer.

3

EVALUATION OF THE IMMUNE SYSTEM AFTER THERAPY DISCONTINUATION IN AN HIV+ PATIENT.

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Background: Evaluation of immune status and viral load of a HIV-1 infected patient after STI for the following 18 months. A 56 years old female, infected in 97, with nadir CD4 of 374 cells/8mu]l and 857 copies/ml, started HAART with a good response (the CD4 counts rose above 1000 and viral load became less than 50copies/ml). In 2002 therapy is stopped due to lipodistrophy. Methods: The study of the

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immune system was performed using 4-color flow cytometry with the following monoclonal antibodies: CD3, CD4, CD8, CD38, CD11a, CD45RO and CD57, in peripheral blood, at 3 different time points (every 6 months). Results: Two months after stopping HAART there was an increase in the viral load (5646 copies/ml) but no decrease in the CD4 counts. Viral load has remained undetectable ever since. CD4 absolute T cells were kept above 1000 cells/μl, with CD4/CD8 ratios around 1. The activation of CD4 and CD8 T cells evaluated by CD38 quantitation showed results similar to uninfected controls, the percentage of naive CD4 and CD8 cells remained constant (median 33% for CD4 and 15% for CD8+ T cells). There was an increase in CD57 expression in CD8+ memory cells from the first to the third time-point (27.5% to 50%). Conclusions: A more detailed phenotype led to the conclusion that, for this time period, the immune system was controlling viral replication, (undetectable viral load and almost no activation of helper and cytotoxic T cells), as well as constant percentages of naive and memory circulating T cells. The increased percentages of CD57 in the CD8+ T cells can possibly be related to an increase in the effector subset. The successful outcome of this patient is probably related to the introduction of HAART soon after seroconversion and the relative preservation of her immune system.

4

CCR7 EXPRESSION IN CONTROLS AND HIV SEROPOSITIVE INDIVIDUALS.

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Background: CCR7 is a chemokine receptor that functions as a homing receptor on migration of naive and memory T cells to secondary lymphoid tissues. The aim of this study was to evaluate the expression of CCR7 in naive and memory CD4+ and CD8+ T cells in healthy donors and in individuals seropositive for HIV-1 without HAART. **Methods**: Whole blood was collected from 6 healthy donors and 13 HIV infected individuals with mean CD4 counts of 720cells/μl (414-1520). Standard 4-color flow cytometry techniques were utilized, a total of 40,000 cells were acquired and the percentage of CCR7+ and CCR7- naive and memory/effector CD4+ and CD8+ T cells obtained. **Results**: HIV+ individuals showed a slight increase in the mean percentage of CCR7+ in the naive subset (CD11adim CD45RO-) in both CD4+ and CD8+ T cells (95,05; 95,23 respectively), compared to healthy subjects (92,45; 93,51 respectively). The values obtained for the memory/effector subset (CD11abright CD45RO+) were 42,44% for CD4+ and 8,10% for CD8+ T cells in patients; in controls, for the same lymphocyte subsets, the values were 42,87% and 9,70% respectively. **Conclusions**: This study shows that CCR7 is expressed in almost all naive cells and has a lesser degree of expression in memory cells, particularly in CD8+ T cells. There was no statistical difference between the 2 groups studied, probably due to low replication of the virus in this cohort of patients. A more detailed analysis to discriminate between memory and effector subsets is recommended.

5

CYTOKINES PROFILES PREVIOUS TO ASSISTED REPRODUCTIVE TECHNIQUES.

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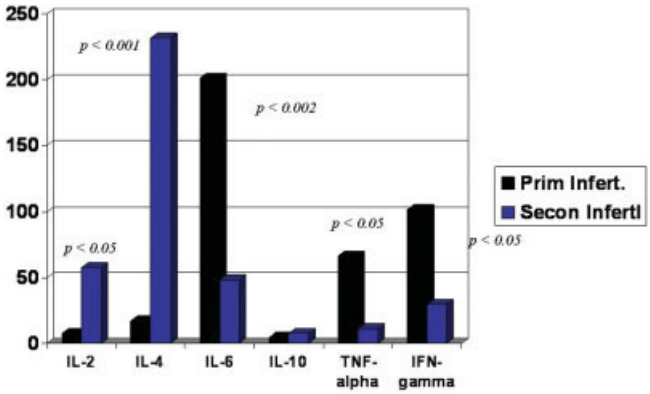
Fertility is modulated by delicate processes of balance between genetic, hormonal, and immunological components. Due to the growing increase of infertility in the feminine population since many years ago, Assisted Reproduction Techniques (ART) have been advanced. To achieve the implantation of an embryo changes in the humoral (Th1 to Th2) and cellular immunological profiles are needed in order to decrease the alloimmune rejection against fetal tissue. Kind of study: This was an experimental descriptive study where cytokines in peripheral blood were analyzed, among a population of infertile patients. Materials and Methods: 95 women diagnosed with infertility were divided in two groups, 26 presenting primary infertility and 69 with secondary infertility. At the moment of the study these patients were starting their menstrual cycle and were not taking contraceptives, hormones, pharmaceutical products or other drugs. A sample of peripheral blood was drawn from each patient in order to obtain plasma in which an analysis of cytokines Th1 / Th2 for CBA (Becton Dickinson) was performed, according to recommendations of the producer. Samples were analyzed in a flow cytometer (FACScalibur BD) and analyzed with CBA software (BD). Results: No significant differences were found among averages of age, number of sexual partners or social-demographic former situations. Patients with primary infertility presented more cases of undergoing ART previously, than patients with secondary infertility. The number of miscarriages in this last group was obviously bigger. The cytokine profiles showed an increase of IL-6, TNF- alpha and IFN-gamma in the group of primary infertility, whereas the group of secondary infertility show an increase of IL-2, IL-4 and IL-6, with diminished levels of TNF-alpha and IFN-gamma. Conclusions: The cytokine profiles in plasma of peripheral blood show that patients with primary infertility have more levels of IL-6 and Th1 cytokines, suggesting that in a basal state they have a strong Th1 stimulus that may explain in part, their infertility. Patients with secondary infertility that show increase of IL-2 and cytokines type Th2 basically pro-inflammatory, suggest that in a basal state they have the Th2 profile that is needed for implantation but are unable to achieve a



Abstract #5: Comparison Of The Plasma Cytokine Profiles Between Primary And Secondary Infertility Patients, Previous To Art

successful term pregnancy where possibly the secretion of inflammatory cytokines by immunological cells, playing and important role in recurrent miscarriage.

6

A NOVEL FOUR-COLOR DIRECT STAINING MULTI-SITE VALIDATION OF ZAP-70 USING A VARIETY OF SPECIMEN TYPES AND PATIENT DIAGNOSIS IN A CLINICAL LABORATORY SETTING.

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We developed and validated a four-color Zap-70 assay (Alexa FluorÆ 488, CALTAG Laboratories) using a surface/cytoplasmic, direct staining technique. Our gating strategy selected CD19+CD5+ CLL cells and evaluated the intracellular Zap-70 expression. Zap-70 expression greater than or equal to 20% was considered positive1. Normal T-lymphocytes present in each sample were used as internal positive control for Zap-70. Our validation included selecting 39 consecutive chronic lymphocytic leukemia (CLL) and small cell lymphocytic lymphoma (SLL) clinical samples submitted to our laboratories in Los Angeles (LA) and New York (NY). Diagnosis was based on morphology and flow cytometry results. A variety of sample types were tested; peripheral blood, bone marrow and tissue. In addition, thirty-four (34) non-CLL/SLL patient samples submitted to our labs were evaluated for Zap-70 when gating on all cell populations present. The Zap-70 qualitative expression agreed with expected marking based on literature on all cases (100% concordance). Five (5) samples (both positive and negative for Zap-70) were split and tested at both our LA and NY facilities. Standardized protocols and flow cytometry instrumentation were used for evaluation. Zap-70 expression agreed qualitatively in both labs with 100% concordance (5/5). Inter- and intra-assay precision studies were performed and qualitative and semi-qualitative results (mean fluorescent intensity) were concordant and within established criteria. Stability studies were also performed with both normal and abnormal samples. We performed an inter-instrument comparison of the same stained sample across multiple instruments with consistent qualitative results. Of the 39 CLL/SLL cases, 25/39 (64%) were negative for Zap-70 and 14/39 (36%) were positive for Zap-70. 13/14 (93%) positive Zap-70 cases were also positive for CD38. Our direct staining Zap-70 assay is specific and sensitive and consistent with published literature. In addition, our assay is less labor intensive than previously published indirect methods. 1. Crespo, M. et al., Zap-70 Expression as a Surrogate for Immunoglobulin-Variable-Region Mutations in Chronic Lymphocytic Leukemia. N Engl J Med 2003 348:18:1764-1775.

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PARALLEL EVALUATION OF COMMERCIAL ANTIBODIES FOR ZAP-70 IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL).

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The expression of Zap-70, a member of the Syk-Zap-70 protein tyrosine kinase family, in CLL cells correlates positively with the mutational status of IgVH, which is associated with poor prognosis. Therefore, Zap-70 expression has been proposed as a surrogate marker for disease prognosis. However, published studies have used specific commercial antibodies and a single strategy for determining expression in CLL cells as a percentage of tumor cells positive for Zap-70, which may not be applicable in all cases. Expression of Zap-70 was determined in 30 CLL patients using four different commercial antibodies. T and NK cells were identified using PE conjugated anti-CD3 and anti-CD56. CLL cells were identified using anti-CD19-PerCP and anti-CD5-APC. The analytical strategy involved using gates for T+NK cells and CLL cells, along with two methods of evaluating Zap-70 expression. Variable Zap-70 expression was observed among the 30 CLL samples. Three antibodies showed wide ranges of Zap-70 expression as a percentage of tumor cells. One antibody showed a high percentage of positive staining of leukemic cells in all 30 samples. The differences may be due to the differences in epitope binding and affinity between different antibodies. NK cells, which are variable in number in CLL specimens, always gave higher Zap-70 expression than T cells. CLL cells gave variable expression from low to equal to NK cells. Significant variation can be introduced by slight changes in the position of the cursor indicating positive T+NK expression. Our results indicate that an analytical strategy, which reports percent CLL cells expressing Zap-70, has poor reproducibility and is not appropriate. Different antibodies give different levels of Zap-70 expression. We propose using a ratio of mean Zap-70 expression in CLL cells to Zap-70 expression in T cells along with a laboratory determined cutoff for elevated Zap-70 expression.

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QASI, AN INTERNET BASED EXTERNAL QUALITY ASSESSMENT PROGRAM FOR CD4 T-CELL ENUMERATION IS AVAILABLE FREE FOR HIV DISEASE MONITORING IN THE GLOBAL VILLAGE.

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Introduction: One of the greatest challenges facing humanity in this century is to arrest the AIDS pandemic. There are some comprehensive efforts ongoing to distribute antiretroviral therapy (ART), in resource-limited regions. Measurement of CD4 T-cell levels is critical for the assessment of HIV disease progression as well as the ART entry criteria. QASI is a Canadian international program for free quality assessment of T-cell subset immunophenotyping performance via internet. **Objective**: WHO has a global commitment to treat 3 million HIV infected individuals by 2005. To meet this urgent global demand for reliable CD4 T-cell enumeration, some capacity building improvements were undertaken by the Canadian program. **Method**: With the support of a multi-level distribution network, stabilized whole blood preparations are shipped free to over 230 laboratories across 50 countries. Aggregate group mean, SD and history performance graphics are generated for each shipment. The 'QASI LymphoSite', a web-based multilingual information system, was introduced in early 2004. **Results**: LymphoSite has improved general accessibility and reduced time cycles, from shipment to performance report publishing, by 20%. The multi-level global distribution network further accelerates response times. Each laboratoryís performance is assessed against all participants and individual reports are issued. The coefficient of variation was reduced between 1997 and 2003. **Conclusion**: Improvements allow rapid and effective multilingual communication hence securing additional time for remedial action if needed. QASI is a unique free service available for monitoring ART to arrest HIV in resource-limited regions of the globe.

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THE CANADIAN EXTERNAL IMMUNOLOGY QUALITY ASSESSMENT PROGRAM (EIQAP) FOR CD4+ T-CELL ENUMERATION.

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Introduction: To halt the AIDS pandemic, clinical trials for drugs and vaccines continue. Monitoring CD4 T-cells remains a critical test for both routine and research. For 15 years, the National HIV Immunology Laboratory (NHIL) has been offering a national EIQAP for CD4 T-cell enumeration to help Canadians living with HIV. **Objective**: To support effective clinical evaluation of new therapies by assuring the quality of CD4 T-cell enumeration across Canada. **Method**: Since 1989, the NHIL has been delivering a Canadian EIQAP for CD4 T-cell determination including national immunophenotyping guidelines and training. Six times a year HIV+ and HIV- fresh whole blood samples are distributed to fifty clinical laboratories. Recently, T-cell subset results are reported using a web based software called 'LymphoSite'. **Results**: The frequency of participation data revealed the impact of the program. It showed a 20% improvement in standard deviation of average accumulated variation for CD4 enumeration after 10 consecutive participations. With the new software LymphoSite, each cycle, from shipment to the performance assessment report, takes less than two weeks. From closing date of data submission, performance reports are issued within 5 days. **Conclusion**: Over time, frequency of participation has a desirable impact on the performance of laboratories. The implementation of the web interface further reduces response time required to generate performance reports. With LymphoSite the time for remedial action has been extended. The Canadian EIQAP for CD4 enumeration continues to provide evidence that it is a didactic and cost effective national service.

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CD8+CLL WITH NEGATIVE ZAP-70 EXPRESSION BY FLOW CYTOMETRY ANALYSIS.

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CLL is the most common leukemia in the Western world with divergent clinical courses. Some patients have indolent disease without need for treatment, others succumb rapidly, and rare patients transform into Richterís syndrome. Although T-cell antigen CD5 is commonly coexpressed, CD8 is unusual in CLL and its expression has not been reported to be associated with consistent morphologic features or clinical behavior. Zap-70 detected by flow cytometry has not been reported in CD8+CLL either. We recently identified a CD8+CLL case with negative CD38. PCR study confirmed B-cell clonality. T-cell receptor gene was germline. Leukemia/lymphoma cells do not express Zap-70 by flow cytometry. IgVH somatic mutation in CLL provides prognostic information. Patients whose leukemic cells express unmutated IgVH often have progressive disease whereas those with mutated IgVH more often have indolent process. Given the availability and technical challenge of IgVH sequencing study, it is unlikely that IgVH sequencing will become a routine clinical laboratory test. Many studies have shown Zap-70 to correlate with IgVH mutation. CLL with unmutated IgVH tend to express higher Zap-70 than those with mutated form. A recent study showed Zap-70 mRNA and protein expression to correctly predict IgVH mutation in 93% CLL patients; its expression and IgVH mutation status were compatible in their ability to predict time to treatment requirement following diagnosis. Since Zap-70 expression can assign most patients to the correct IgVH mutation status, measurement of Zap-70 could be a useful serrogate for this distinction. To our knowl-

edge, this is the first reported CD8+CLL case with Zap-70 negativity by flow cytometry analysis. Whether Zap-70 provides similar prognostic information in CD8+CLL patients remains uncertain. Correlation of Zap-70 with IgVH mutation in CD8+CLL requires confirmation. Its significance in predicting disease course in this subgroup also requires further study.

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COMPARISON BETWEEN IMMUNOPHENOTYPIC ASSESSMENT OF SURFACE AND CYTOPLAMIC LIGHT CHAIN RESTRICTION IN B CELL LYMPHOPROLIFERATIVE DISORDERS.

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BACKGROUND: Immunophenotypic detection of light chain restriction by flow cytometry (FC) represents evidence of clonality of B cells and assists in diagnosis of B cell lymphoproliferative disorders. Therefore, obtaining an unequivocal light chain restriction is important. Traditionally FC has assessed surface immunoglobulin expression, however, given that κ and λ light chains are expressed intracellularly from immature B cell stages to terminally differentiated plasma cells, unlike the expression on the surface, cytoplasmic staining may be more appropriate in detecting clonality. **OBJECTIVE**: To compare and contrast the sensitivity, specificity of cytoplasmic detection of κ/λ to surface staining and determine its role in routine clinical laboratory practice. **DESIGN**: 97 samples (30 bone marrow, 23 peripheral blood, 29 lymph node, 9 pleural fluid, 6 tissue mass) suspected of harboring B cell lymphoproliferative disorders were collected prospectively. The samples were analyzed in parallel for the expression of surface and cytoplasmic κ/λ using polyclonal anti-κ and anti-λ light chain antibodies. Diagnosis was confirmed by morphology and when required PCR based IgH or bcl-2 gene rearrangement. 20 peripheral blood samples were diagnosed by FC exclusively. A κ/λ ratio outside the range of 0.9-3.0 was regarded as the existence of clonal B-cells. **RESULTS**: 49 of the 97 samples had confirmed B cell lymphoproliferative disorders (2 PTLD, 13 CLL, 34 NHL including FL(14), MZL(6), LDL(3), SLL(3), DLBCL(3), BL(1), MCL(1), mixed small and large cell lymphoma(1) and NOS(2)). There was 87% agreement between surface and cytoplasmic techniques. However, statistical analysis showed 86% sensitivity, 75% specificity, 78% PPV, 84% NPV with surface staining and 92% sensitivity, 79% specificity, 82% PPV, 91% NPV with cytoplasmic staining. Although the sensitivity increased to 96% when the data from both techniques were combined and light chains from either method were reported as monoclonal, there was a decrease in specificity to 71%. **CONCLUSION**: Cytoplasmic staining rather than surface staining of κ/λ light chains may be a preferred technique to detect monoclonality in B cell lymphoproliferative disorders by FC.

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A MULTI-INSTITUTIONAL STUDY EXAMINING FLOW CYTOMETRIC ASSESSMENT OF ZAP-70 KINASE EXPRESSION IN NORMAL AND CLL LYMPHOCYTES.

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The current 'state-of-the-art' for the flow cytometric assessment of Zap-70 kinase activity in CLL relies on the use of percentage as a reporting statistic. This statistic is derived by comparing the expression in CLL cells (CD5+/CD19+) with other co-habitant lymphocyte subsets exhibiting demonstrable expression of the epitope. We undertook a multi-institutional study in which four sites analyzed split samples from normal (N=20) and presumptive CLL (clonal, CD5+, B-cell lesions; N=45). Zap-70 kinase expression was examined using each institution's intracellular antigen staining protocol without attempting methodological uniformity. Three lymphocyte subsets (CD5+/CD19-, CD5-/CD19+, CD5-/19-) were evaluated in the normal cohort. In the CLL cohort, the same three subsets were evaluated, in addition to assessing Zap-70 kinase and surface membrane CD38 expression in the CD5+/CD19+ subset. Our working hypothesis is that use of the 'percent positive', derived by comparison with the internal CD5+/CD19- 'control' population, by itself is not an appropriate statistic due to technical, as well as biological variability. We will present data to support our contention that fluorescence quantitation, using fluorescence equivalents, antibody binding sites, and/or intensity ratio (compared to autofluorescence background) is the preferred evaluation parameter. The ability to resolve categories of reactivity may be enhanced by simultaneous evaluation of fluorescence quantitation and percentage statistics. We also found no correlation between Zap-70 kinase and CD38 expression in CD5+/CD19+ lymphocytes from CLL samples.

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CHARACTERIZATION OF CD146 EXPRESSION ON PERIPHERAL BLOOD LYMPHOCYTES.

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CD146, also known as P1H12, s-Endo-1, MUC18, and Mel-CAM, is a cell adhesion molecule involved in cell-cell interactions. CD146 positive cells comprise approximately 0.2% to 1.5% of the peripheral blood mononuclear cells. CECs are identified by flow cytometry as CD146+, CD45-, although numerous studies have relied solely on CD146 magnetic bead separation for purification of CECs. A previous report (ref) identified CD146 on T cells after in vitro stimulation with PHA. In our current study, we have demonstrated the presence of CD146 on B and T cells in fresh peripheral blood and have further explored the upregulation of this antigen after in

vitro stimulation. Of the CD146 positive cells found in the peripheral blood, a high percentage are CD45 positive. These CD146, CD45 dual positive cells in fresh peripheral blood are predominantly (93%) CD3+. Less than 1% of the CD3+ T cells from peripheral blood express CD146 in the normal subjects studied. Both CD4 and CD8 T cells may express CD146, although most subjects display more CD4, CD146 dual positive cells than CD8, CD146 dual positive cells. CD146 expression on T cells does not correlate with expression of HLA-DR, CD69, and CD25, although there is some co-expression of these activation markers. An increase in the percentage of T cells expressing CD146 is observed after 72 hours in vitro stimulation with PHA. Using CSFE, it was determined that CD146 expression increased with every cell division. A low percentage of B cells also co-expressed CD146 in fresh peripheral blood, and it is currently under investigation to determine if this can be upregulated by in vitro activation. We conclude that CD146 expression in peripheral blood is not limited to CECs and is found on several lymphocyte subsets.

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CASE STUDY: FOUR-COLOR FLOW CYTOMETRY IDENTIFIES B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CASE WITH TWO DISTINCT CLONES OF ZAP-70 POSITIVE AND NEGATIVE NEOPLASTIC B-LYMPHOID CELLS.

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We present a case of a peripheral blood submitted to us for Leukemia/Lymphoma Evaluation by flow cytometry. The patient is a 66 year old male with an 8 year history of CLL with both kappa and lambda positive CLL clones identified in 1996 (kappa: lambda equaled 3:1). A four-color flow cytometric evaluation was performed. A population of 88% lymphoid cells was identified by CD45 vs. SS gating. A complete phenotypic evaluation of the lymphoid population was performed with both and an open-gate strategy and a CD45 vs. SS gating strategy. All populations present were evaluated for phenotypic expression as a quality control. We identified two distinct malignant B-cell lymphoid populations. Each population had different phenotypic characteristics. The predominant B-lymphoid population (45% of lymphoid cells) dimly expressed monoclonal kappa light chain and also the following markers: HLA-DR, CD5, CD11c, CD19, CD22, CD23 and CD45. They were clearly CD38 and Zap-70 negative. The second population (33% of the lymphoid cells) dimly expressed monoclonal lambda light chain and also the following markers: HLA-DR, CD5, CD11c, CD19, CD22, CD23 and CD45. Most significantly, this secondary minority clone was clearly CD38 and Zap-70 positive. Overall, if the two populations were not separately analyzed, the kappa: lambda ratio of the entire B-cell population would fall within the normal range of (equaled 1.36; normal in our laboratory is approximately 1-2, with the ratio equaling 1.5 in most clearly benign cases). This case clearly demonstrates the importance of correct identification of more than one abnormal B lymphoid clone. In this particular case, if the populations had not been separately evaluated for Zap-70 and CD38 expression, the more aggressive nature of the lambda positive clone might have been missed. In some CLL cases, especially those with more than one clone, reagent combinations that evaluate CD38 and Zap-70 expression for the entire CLL population may even yield results that are prognostically misleading.

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IMMUNE MONITORING WITH iTAg&Oχιρχ; MHC TETRAMERS: PREDICTION OF RECURRENT CMV REACTIVATION IN ALLOGENEIC STEM CELL TRANSPLANT RECIPIENTS.

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This study evaluated the use of tetramers in monitoring CMV-specific T-cell recovery following allogeneic stem cell transplantation (SCT) to predict patients at risk for CMV-related complications. SCT recipients were monitored for up to 944 days for viremia, lymphocyte counts, and clinical status. iTAg MHC Tetramers (Beckman Coulter, San Diego) were used to enumerate CMV-specific CD8 T-cells by flow cytometry using a single-platform absolute counting method. The following tetramers were included: pp50: A*0101 (VTEHDTLLY); pp65: A*0101 (YSEHPTFTSQY), A*0201 (NLVPMVATV), A*2402 (QYDPVAALF), B*0702 (TPRVTGGGAM), B*3501 (IPSINVHHY); pp150: A*0301 (TVYPPSSTAK); IE-1: A*0201 (VLEETSVML), B*0801 (ELRRKMMYM). Data were analyzed for 36 myeloablated CMV-seropositive patients with early tetramer values available (0-100 days). Reduced intensity regimens will be analyzed separately when adequate follow-up times have been obtained. Study results indicate that myeloablated patients with a slow rate of recovery of CMV-specific T-cells (unable to mount a response above 2 cells/μl in the first 60-100 days post transplant) were at risk for complications. Patients with a maximum response < 2 cells/μl were 3.8 times more likely to have recurrent viremia ($p = 0.01$). Of interest, this patient group was also 2.5 times more likely to develop extensive cGVHD ($p = 0.03$), and 3 times more likely to develop non-relapse fatal complications ($p = 0.004$). Tetramers have potential clinical utility in monitoring CMV-specific T-cells to predict patients at risk of CMV-related complications, allowing clinicians to further refine pre-emptive therapeutic strategies in appropriate high-risk populations, and possibly in the future to monitor pre- and post- antigen-specific cellular immunotherapy.

16

ASSESSMENT OF CD52 STATUS BY FLOW CYTOMETRY IS FUTILE PRACTICE PRIOR TO INITIATION OF CAMPATH-1H TREATMENT FOR CHRONIC LYMPHOCYTIC LEUKEMIA.

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CAMPATH-1H (Alemtuzumab), a humanlized monoclonal antibody directed against CD52, has been an alternative therapy for treating chronic lymphocytic leukemia. Little is known about the frequency and variation in antigen intensity of CD52 expression in chronic lymphocytic leukemia. It is not clear that whether assessment of CD52 antigen status is necessary prior to initiation of CAMPATH-1H therapy. We examined 100 consecutive cases chronic lymphocytic leukemia with four color multiparameter flow cytometry at OHSU. The immunophenotypic features were studied with a panel antibodies against CD5, CD10, CD19, CD20, CD22, CD23, CD38, Kappa and lmabda. The expression of CD52 was evaluated in gated leukemic cells expressing both CD5 and CD19. CD52 expression was compared between CLL cases positive for CD38(> or = 30%) and CLL cases negative for CD38(<30%). In addition, we investigated whether there was a correlation of CD52 expression with cytogenetic abnormalities in cases stuided by FISH with probes for trisomy 13, deletion of p53, ATM and RB1. We found that CD52 was relatively homogenously expressed in luekemic cells in all cases. The antigen intensity of CD52 ranged from 1 to 2 logs greater than isotypic background control among the case tested. The CD52 antigen expression was not affected by antigen intensity of CD19 or CD20. There was no association between CD52 antigen intensity and CD38 expression. No difference of antigen intensity was detected among cases with normal cytogenetics and cases with trisomy 13, deletion of p53, ATM, or RB1. Therefore, we concluded that assessment of CD52 status is unnecessary as a routine practice for chronic lymphocytic leukemia prior to initiation of CAMPATH-1H therapy.

17

AN ALTERNATIVE METHOD TO [3H]-THYMIDINE ASSAY FOR THE ASSESSMENT OF LYMPHOCYTE MITOGEN STIMULATION BY FLOW CYTOMETRY.

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The most widely used in vitro measure of T-cell function has been the assessment of mitogen induced proliferation by [3H]-thymidine incorporation. This lengthy method requires the use of radioactive nucleotides and does not provide information about functional reponses of individual lymphocyte subsets. In our flow cytometric method, isolated lymphocytes are set up in culture for three days and challenged with 3 different T cell stimulating mitogens: phytohemagglutinin (PHA), concanavalin A (ConA) and pokeweed mitogen (PWM). After the incubation period, the lymphocytes are harvested and stained cytoplasmically to assess the expression of the proliferative antigens Ki-67 (nuclear protein in all multiplying cells) and PCNA (proliferating cell nuclear antigen) to determine the percentage of cells in growth phase. In addition, cells are counted to determine the increase in cell number due to the activation and subsequent proliferation of cells in response to the mitogens. Co-staining with CD3 (T cell marker) and CD19 (B cell marker) enables the measurement of specific cell types. The method is simple, reliable, non radioactive and enumerates as well as differentiates the cell proliferation response. This facilitates the comparison between different patients and allows easy monitoring and follow up of known immunodeficient patients under treatment.

18

GUAVA TECHNOLOGIES EASYCD4™ AND EASYCD8™ ASSAYS *VS.* BD BIOSCIENCES MULTITEST™ ASSAY FOR THE ENUMERATION OF CD4+ AND CD8+ T CELLS : THREE-SITE EVALUATION.

Steven Josefowicz [1], Kfir Elad [2], Eileen Liu [3], Okumu K'Aluoch [3], Kimvan Tran [4], Kamala Tyagarajan [4], Leonard Buchner [4], Haynes Sheppard [3], Martin Bigos [1], Lorrie Epling [2], Elizabeth Sinclair [2], Barry Bredt [2].
[1]Gladstone Institute of Virology and Immunology, [2]University of California, San Francisco, [3]California Department of Health Services, [4]Guava Technologies, Inc.

Design: Performance of EasyCD4 and EasyCD8 assays (Guava Technologies) assays at three sites was evaluated *vs.* the predicate MultiTest assay (BD Biosciences) performed at the UCSF Clinical Laboratory. With informed consent of all volunteers, EDTA-anti-coagulated whole blood specimens (n=74) from HIV-1-seronegative and -seropostive donors were collected and tested by EasyCD4 and EasyCD8 in triplicate at three sites. In addition, a tube of matched blood was analyzed by the predicate method at the UCSF Clinical Laboratory. **Results:** High correlation was observed between EasyCD4 and EasyCD8 *vs.* predicate over the entire range tested (CD4 range = 1-1417 cells/mL, median = 544 cells/mL; CD8 range = 101-2082 cells/mL, median = 496 cells/mL). EasyCD4 and EasyCD8 *vs.* predicate percent difference plots showed no trends in bias across the observed range. The median percent differences for the 3 sites ranged from -1.8%-8.3% (EasyCD4) and 13.1%-8.6% (EasyCD8). Inter-site variability was 10.6% (EasyCD4) and 10.8% (EasyCD8). Intra-site variability was 4.1%-5.6% (EasyCD4) and 4.5-5.1% (EasyCD8) [Kruskal-Wallis statistical test]. **Conclusions:** EasyCD4 and EasyCD8 assays provide excellent accuracy and precision in the range ~1-2000 cells/mL. Correlation and agreement of EasyCD4 and EasyCD8 with the predicate assay, and inter- and intra-site assay reproducibility were excellent. A small negative bias (~10%) between EasyCD8 and MultiTest assay was observed. The EasyCD4 and EasyCD8 assays thus represent accurate, precise, simple and low-cost alternatives to traditional methods for CD4+ and CD8+ T cell enumeration.

19

MULTIPLEX BEAD ARRAY ASSAYS FOR DETECTION OF SOLUBLE CYTOKINES: COMPARISONS OF KITS FROM MULTIPLE MANUFACTURERS.

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Background: Multiplex bead array assays permit simultaneous cytometric quantitation of multiple cytokines in solution by capturing these to spectrally distinct beads. Since several manufacturers offer reagents to quantitative the same cytokines on a single instrument, a comparison should be made to determine if these kits yield similar data and whether these data are comparable to ELISA. **Methods**: This study was designed to compare cytokine detection kits using the Luminex 100Æ. Twenty-six serum samples from seven subjects were analyzed for IFNγ, IL-1β, IL-6, IL-8, and TNFα, using multiplex kits from LINCO Research, Bio-Rad Laboratories, R&D Systems, and BioSource International. Each assay was performed according to the manufacturers' specifications. Standard curves were generated using reference concentrations supplied by each manufacturer. ELISAs for IL-8 were performed using kits from R&D and BioSource. **Results**: Cytokine levels followed similar patterns, although absolute concentrations differed among kits. ELISA and Luminex values for IL-8 were similar in kits from the same manufacturer. **Conclusions**: Since relative cytokine measurements are often valuable when performed serially, it may be possible to make inter-lab comparisons using different kits. When comparison of absolute values is crucial, kits from the same supplier should be used. Within vendor, bead array and ELISA values appear comparable.

20

ELECTRONIC NUCLEAR VOLUME AND DNA CONTENT OF HUMAN DUCTAL CARCINOMA IN SITU BREAST TUMORS.

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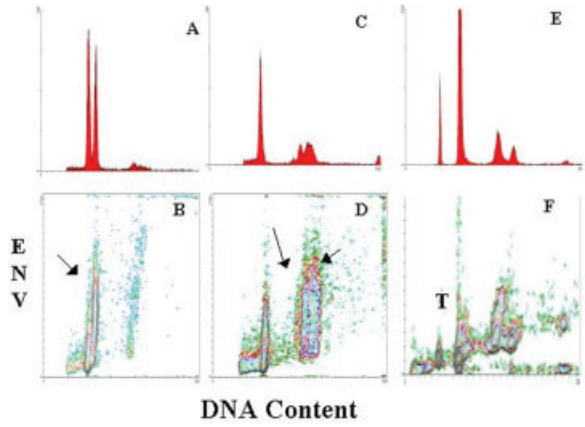
University Of Miami School Of Medicine.

Nuclear size and chromatin texture are used as important parameter in histopathological grading of human breast ductal carcinoma in situ (DCIS). Most of these histopathological studies are carried out visually under a microscope and thus are slow and often based on examination of a small number of cells. Although electronic scanning or morphometry has been used in a few studies to measure nuclear size in breast tumor sections, most of these methods have not found wide acceptance. In contrast monitoring of electronic nuclear volume by flow cytometry could be a rapid method for determination of nuclear volume and possibly for rapid grading of DCIS. We have earlier described the development and application of the NASA/ACS flow cytometer that can measure electronic volume and DNA content of nuclei isolated from fresh or paraffin embedded tumor (Cytometry 43: 2-11, 2001). We have shown that simultaneous measurement of electronic volume versus DNA content can be used to identify sub-populations in a heterogeneous population and possibly discriminate between normal and tumor cells in solid tumors (Cytometry 43: 16-22, 2001). In the present study, we have used a NPE Quanta flow cytometer (a commercial and advanced version of the NASA/ACS flow cytometer manufactured by NPE Systems, Pembroke Pines, Fl.) for monitoring of nuclear volume and DNA content of DCIS. Nuclei from cryopreserved biopsies were disaggregated in DAPI staining solution (Cytometry, 43: 12-15,2001) and trout red blood cells were added as an internal standard. Data will be presented to show that this rapid method can identify and discriminate between diploid and aneuploid DCIS which differ in their nuclear volumes.



Abstract #20: Electronic nuclear volume vs. DNA content of DCIS

21

THE USE OF LANTHANIDE(III)-MACROCYCLES, QUANTUM DYESÆ, FOR CELL CYCLE MEASUREMENTS.

Robert Leif [1], Margie Becker [2], Al Bromm, jr. [3], Lidia Vallarino [3], Sean Yang [1&2].

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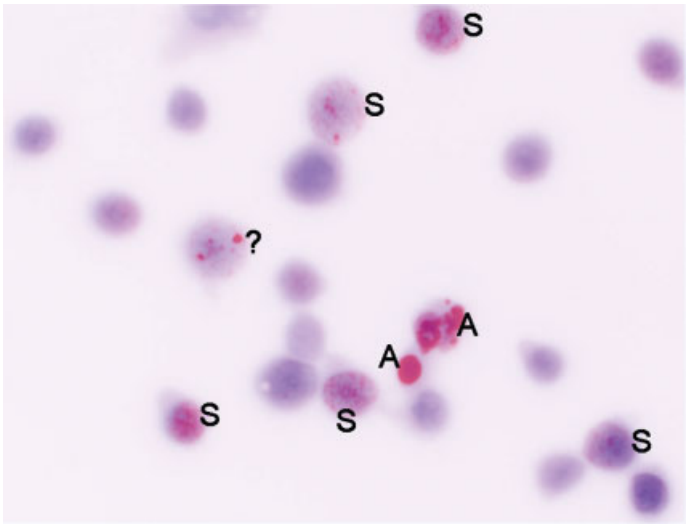
The high signal-to-noise level of luminescent macrocyclic complexes of lanthanide (III) ions, Quantum DyesÆ, lack of concentration quenching of their emissions, and their small size (1/224 of a QdotÆ), make them ideal for use as tags for antibodies against nuclear antigens, allow their simultaneous employment with other labels, and provide future use as tags for nucleic acid probes. Direct preparations of both apoptotic and S phase cells have been obtained using the europium Quantum Dye conjugate of anti-5-BrdU, with DAPI counter-staining. Under these conditions, it appears that the islands of DNA synthesis can be morphologically distinguished from apoptotic bodies. The analysis of DNA histograms can be simplified by prior removal of S Phase and apoptotic cells from the DNA distribution. The emission intensity of the conjugates was increased by energy transfer



Abstract #21: S phase, apoptosis, and DNA

from a complex of a non-luminescent lanthanide ion to the Quantum Dye. The use of a dry mount has resulted in an increase of the luminescence emission, as well as less UV light induced fading. The narrow emission of the europium macrocycle (5.2 nm width at half-maximum) and the large Stoke's shift (246 nm) permit the elimination of the long wavelength tail of the DAPI emission. The long lifetime (1 msec) of the europium emission also permits the total elimination of the DAPI emission by the use of simple time-gating. An enhancer has been found that enables the observation of simultaneous emissions from both the europium and terbium macrocycles. A computer program has been developed to transform fluorescence and luminescence images into conventionally appearing (white background) absorbance images, which are familiar to pathologists and other medical personnel. This combination of new stains and software affords an opportunity to replace the present 19th century hematoxylin (1865) and eosin (1871) stains still employed in pathology with modern stains based on molecular biology.

22

CYTOMETRYML, A NEW DATA STANDARD FOR CLINICAL CYTOMETRY.

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Background: CytometryML is a new XML based standard that has been created for the storage and manipulation of clinical flow and image cytometry data. A requirements document and hazard analysis, which are required by the FDA, have been created and published. The use of XML greatly simplifies interfacing with hospital information management systems, including the data produced by Health Level 7, and medical picture achieving systems. CytometryML describes a list mode file in the form of a Digital Imaging and Communications in Medicine standard (DICOM) Waveform Information Object. Method: The XML data types were modeled on the corresponding DICOM data types, structures, and information objects and were described in XML schema. The binary image and list mode data are kept in files that are separate from the XML documents. The images can be stored in standard formats: JPEG, Tiff, etc. However, the suggested format is AdobeÆ PhotoshopÆ RAW, which permits direct access to the image. The flow data is also stored as a simple object; and data to facilitate the reading of these data files is included in the XML page. Results: It was possible to express many of the DICOM data types in XML schema. The Waveform Information Object representation of the list mode information was a significant improvement over the original. The collection of XML schemas and pages is programming language independent and is consistent with governmental standards including those of the USA. The use of a RAW image format facilitated the development of a program that transforms fluorescence images into conventionally appearing (white background) absorbance images, which are familiar looking to pathologists and other medical personnel. Conclusion: CytometryML is a standard suitable for clinical cytology and should be accepted as the replacement for Flow Cytometry Standard (FCS). CytometryML is also a more efficient format for data analysis.

23

VALIDATION OF LYMPHOCYTE IMMUNOPHENOTYPING USING THE MULTITEST IMK ASSAY.

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Using whole blood samples from healthy volunteers, the MultiTEST IMK Assay with TruCOUNT beads was validated and the biological variability of lymphocyte subsets evaluated. In addition, the stability of T lymphocytes in HIV-positive whole blood samples, the dilution effect of liquid anticoagulants on absolute counts, and the stability of lymphocyte subsets in preserved whole blood were assessed. Intra- and inter-assay precision for lymphocyte subset absolute counts were 2.8-7.0 %CV and 5.5-9.0 %CV,

respectively. Intra- and inter-assay precision for lymphocyte subset relative percentages were 0.8-5.0 %CV and 1.6-9.3 %CV, respectively. When whole blood samples were diluted in plasma, good linearity was demonstrated (R2 >0.9993). Intra-subject lymphocyte subset values from 46 healthy volunteers varied between 3.2 to 22.5 %CV when analyzed over a two-month period. The stability of lymphocyte samples from healthy volunteers collected in either ACD, EDTA or Na++ heparin was assessed. There was less than a 9% change from baseline in the ACD samples held at room temperature for 72 hours. T lymphocyte subsets in ACD whole blood samples collected from HIV-positive subjects also demonstrated good stability up to 72 h post-collection. A comparison of samples collected in ACD, EDTA, and sodium heparin, indicated that ACD was the preferred anticoagulant for samples stored for 24 to 72 h. However, ACD tubes contain a liquid anticoagulant and assay results for ACD samples must be adjusted to correct for that dilution. Processed samples stored in FACS Lysing Solution were stable for 48 h at RT or 4C. In addition, whole blood preserved with Cyto-Chex Reagent demonstrated good stability up to 7 days post-collection.

24

ANALYTICAL PERFORMANCE OF iTAg&Oχιρχ; MHC CMV TETRAMERS* IN MEASUREMENT OF CMV-SPECIFIC T CELLS IN WHOLE BLOOD.

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Enumeration of antigen-specific T cells for Cytomegalovirus (CMV) has potential clinical utility in monitoring allogeneic stem cell transplant recipients. To monitor the clinical course of disease, measurements must be sensitive, accurate and reproducible. We evaluated the performance of Beckman Coulterís Class I iTAg&Oχιρχ; MHC CMV Tetramers for enumeration of CMV-specific CD8 T-cells. The method used was a single-platform, 2-panel technique. CD3+CD8+ cells were enumerated using anti-CD3 and -CD8 antibodies and Flow-Count&Oχιρχ; Fluorospheres in a lyse-no-wash method. The frequency of tetramer positive cells was determined using anti-CD3 and -CD8 antibodies and tetramer in a lyse-with-wash method. The two results were combined to yield the absolute count of CMV-specific CD8+ T-cells. Reproducibility was evaluated using EDTA anticoagulated whole blood from nine healthy donors and HLA-A*0101, A*0201, and B*0702 CMV tetramers. Samples were run in replicates of 10 and CVs ranged from 1.3 to 8.0% for tetramer results between 2 and 30 cells/μL. Interlaboratory precision across 3 labs and operators ranged from 3.2 to 13.3%. Evaluation of variability between 3 lots of each tetramer resulted in CVs ranging from 0.4 to 6.4%. Sensitivity and linearity were determined by titrating cells from CMV-specific CD8+ T-cell clones into whole blood from a CMV tetramer negative donor. Dose response curves were linear up to 90 cells/μL and the functional sensitivity was 1.0 cell/μL. Equivalent results were generated on whole blood from eighteen healthy donors using a Beckman Coulter EPICS&Oχιρχ; XL and a Becton Dickinson FACSCalibur&Oχιρχ; with a correlation coefficient of 0.9980 and slope of 0.9705. Enumeration of CMV-specific CD8+ T-cells using iTAg&Oχιρχ; MHC CMV tetramers was shown to have good reproducibility, sensitivity, and linearity sufficient to support its use in monitoring clinical patients.

**For research use only. Not for use in diagnostic procedures.*

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CD10 EXPRESSION ON PLASMA CELLS: MYTH OR REALITY?

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A review of the literature shows that plasma cell immunophenotypes have largely been determined based on 2 or 3 colour flow cytometry by gatin on CD38++ events. Plasma cells in bone marrow samples have been reported as CD10+ in as many as one-third of malignant cases. As a result, differential diagnosis on a CD10 positive clonally restricted cell includes plasma cell dyscrasias as well as follicular lymphoma, diffuse large cell lymphoma, Burkittís lymphoma, hairy cell lymphoma, precursor B-lymphoblastic lymphoma and myeloproliferative disease. The objective of this study was to utilize 5 colour flow cytometry to determine the presence or absence of CD10 on malignant plasma cells using a multicolour gating strategy to more specifically identify malignant plasma cells from other bone marrow components including hematogones which are known to be CD38++ and CD10+. Staging bone marrows (n=49)which were confirmed by flow cytometry, morphology and/or molecular techniques were immunophenotyped using CD38++, CD45 dim/- and CD138+ as gating markers to assess expression of CD10 on plasma cells and also to exclude normal CD10+38++ hematogones from analysis. In all 49 cases, no evidence of CD10 expression on malignant plasma cells was seen when CD38++ hematogones were excluded from the gating criteria. In conclusion, CD10 expression on plasma cells is either extremely rare or non-existant and previous reports of CD10+ malignant plasma cells may be a result of using CD38++ gating to identify plasma cells for immunophenotyping purposes. This finding should help subclassify CD10+ proliferations since CD10 is more commonly found in non-plasma cell dyscrasias.

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CASE REPORT: T-ALL AS A SECOND MALIGNANCY IN A PATIENT WITH CLL.

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A 77 year old male with a leukocytosis of 114.0 x 10^3 /uL and lymphocytosis of 86.0 x 10^3 / uL was diagnosed as B-CLL that responded well to alkylating agents and prednisone. Flow cytometry at diagnosis showed that most of the lymphocytes were B cells co-expressing CD19, CD5, Kappa (dim), CD23, CD20 (dim) and negative for FMC7. A small normal T cell population was also present. Two years later, the patient had a recurrence of a high leukocyte count with peripheral lymphocytosis. As he did not respond this time to a repeat course of leukeran and steroids nor to fludarabin, a repeat flow cytometry on peripheral blood was done. The CLL clone was clearly present but made up less than 1% of the total lymphocytes. The remaining cells were compatible with T lymphoblastic leukemia. Despite chemotherapy the patient died within 6 weeks. Conclusions: 1. Flow cytometry of peripheral blood of CLL patients should be part of the patient follow-up. An increase in lymphocytes should not be automatically interpreted as a relapse or variant of the original B cell clone. 2. The relative rarity of T-ALL as a second malignancy in CLL, as deduced from the literature, is in line with its rarity within the spectrum of malignant diseases in the general population and should not be construed as a special feature of CLL.

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METHODOLOGICAL ASPECTS OF ZAP-70 EXPRESSION IN PERIPHERAL BLOOD OF HEALTHY VOLUNTEERS AND PATIENTS WITH CD5 POSITIVE LYMPHOPROLIFERATIVE DISORDERS.

Debra Marlott, Carol Bolinger, Marcia Doty, Tony Ginn, Norma Redmond, Attilio Orazi, Magdalena Czader.

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Zeta-associated protein 70 (ZAP-70), member of Syk family, has been originally reported to be involved in T-cell receptor signaling and recently shown to play a role in pro-B to pre-B cell transition. The expression of ZAP-70 in chronic lymphocytic leukemia (CLL) correlate with unmutated immunoglobulin heavy-chain variable region genes and with prognosis. In this study, using directly conjugated antibody clone 1E7.2, we investigated the expression of ZAP-70 in B-, T- and NK-cells from peripheral blood (PB) of healthy volunteers and patients with CD5 positive lymphoproliferative disorders. Nineteen normal PB samples and 15 CLL/SLL and mantle cell lymphoma (MCL) specimens were studied. Normal samples were processed and analyzed on the day of collection (day 1), day 2 and after PFA fixation to mimic a variety of real-life scenarios. The CD3+/CD56+ cells were used as an internal control. Percentage of positive cells, median fluorescence intensity (MFI) and relative MFI (RMFI) were calculated. A significant proportion of normal B-cells expressed ZAP-70 (mean 71.2%, SD 12.1), although at lower intensity levels than CD3+/CD56+ cells (mean RMFI 6.7 and 14.0 for B- and T/NK-cells, respectively). NK-cells showed significantly higher ZAP-70 intensity than T-cells. As expected, samples analyzed after PFA fixation showed lower MFI than those analyzed on day 1. There was a statistically significant difference between ZAP-70 expression on day 1 and day 2, with the latter showing lower intensity of staining for all cell types. Three patterns of ZAP-70 expression were observed in patient samples: 1) definite positive with intensity comparable to that of T-cells (8 cases), 2) definite negative with intensity significantly lower than internal T-cell control (1 CLL/SLL, 2 MCL) and 3) equivocal with heterogeneous ZAP-70 expression (2 CLL/SLL and 1 MCL). The interpretation of ZAP-70 expression in the latter group may be difficult and highly variable considering significant variation of ZAP-70 intensity dependent on processing.

28

EFFECT OF BLOOD STORAGE TEMPERATURE ON ABSOLUTE CD4 AND CD8 COUNTS.

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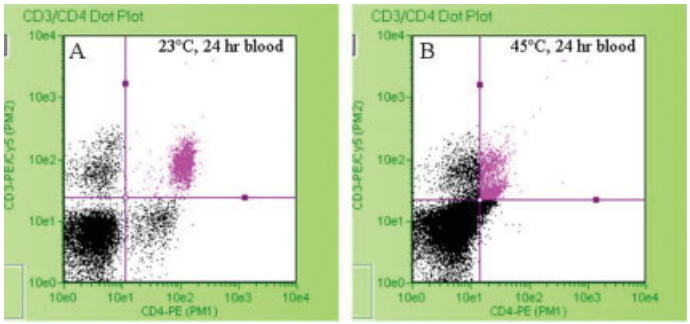
Guava Technologies, Hayward, CA 94545.

Absolute CD4 and CD8 counts have become an essential component of anti-retroviral therapy and HIV monitoring. While several well-established methods exist for CD4 and CD8 counting, little attention has been focused on factors that can impact counts such as blood storage temperature. Earlier studies have not evaluated the effect of storage times at elevated temperatures, which is relevant to countries where HIV/AIDS prevalence is high and where temperatures often exceed 40°C in the summer. Here we present results from a controlled study where impact of blood storage temperature on CD4 and CD8 counts was monitored using the EasyCD4 and EasyCD8 assays on the Guava PCA, a micro-capillary flow cytometer. Aliquots of blood from normal donors were incubated at 4°, 23°, 37° and 45°C for various periods of time prior to determining CD4 and CD8 counts. Our results demonstrate that there is a significant deterioration in both CD4 and CD8 counts when blood is stored at 45°C for just 2 hrs. The average percent difference for blood samples stored for 2 hrs at 4°C, 37°C and 45°C for CD4 counts was ñ2.8. -0.9 and -19.6% respectively, while the % difference in CD8 counts was 2.1, ñ1.5 and -19.3% respectively when compared to the counts from samples stored at 23°C. On longer storage times at 45°C, the staining profiles are completely deteriorated and cannot be used for reliable counts. Our results suggest that transferring blood to 23°C or 4°C or the addition of blood stabilizers to samples at elevated temperatures, must be performed soon after draw to obtain accu-



Abstract #28: Impact of Blood Storage Temperature on CD3/CD4 Staining: (A) Blood at 23°C for 24 hrs and (B) Blood at 45°C for 24 hrs

rate absolute CD4 and CD8 counts in resource-poor settings where ambient temperatures exceed body temperature.

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A RAPID, SIMPLE BEAD-BASED ASSAY TO VALIDATE BIOCONTAINMENT IN A CELL SORTER.

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Sorting of infectious agents and/or potentially infectious human cells with high-speed cell sorters leads to significant biosafety concerns. Validation of biocontainment should be standard operation. The bacteriophage T4 assay is a sensitive measure of aerosol containment but technically challenging (Schmid, et al, Cytometry 28:99-117, 1997). Also, the necessity to culture the phage delays the result of the test and may compromise safety. Recent protocols have used Glo GermÆ, an infection monitoring particle, to visualize failure of aerosol containment, either alone (Oberyszyn and Robertson, Cytometry 43:217-222, 2001) or with an aerosol collection system to increase the sensitivity (Perfetto, et al, Cytometry 52A: 122-130, 2003). Although intensely fluorescent, Glo GermÆ particles are suspended in an oil-based medium and require preparation before use. We present a practical alternative using Polysciences YG beads and a sensitive air-sampling device. Poly YG beads (0.50- 20μ) were compared with Glo Germ (<5μ) as markers for aerosol escape from a FACSDiVa with aerosol management in a BSL3 environment. Sampling was performed using the cyclex-dÆ system (Environmental Monitoring Systems) for 10 minutes directly in front of the closed sort chamber, before and after an ìartificial clogî. Escaping beads, captured onto a coverslip were counted using a fluorescent microscope. Simulating worse case conditions resulted in a range of 0-11 (mean 1.6) Poly YG beads and a range of 0-15 (mean 4.3) Glo Germ beads counted on the cyclex-d coverslip. This new approach, with sensitivity similar to Glo GermÆ, offers a practical approach to evaluation of biocontainment on cell sorters.

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TCR-Vβ EXPRESSION BY FLOW CYTOMETRY: COMPARISONS WITH TCR GENE REARRANGEMENT USING CAPILLARY GEL ELECTROPHORESIS FOR ESTABLISHING CLONALITY.

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Flow cytometric analyses of human T-cell receptors (TCRs) using TCR β-chain variable region families (TCR-Vβ) constitutes an alternative to traditional molecular techniques for the detection of T-cell clonality. Thirty-three patient specimens were tested for TCR gene rearrangement (TCRγ, TCRδ, and/or TCRβ) (InVivoscribe Technologies, San Diego, CA) and analyzed using capillary gel electrophoresis (CGE) with fragment length analyses (ABI 310 Genetic Analyzer, Applied Biosystems, Inc., Foster City, CA). Nineteen cases (56%) showed clonal rearrangements: 18 cases were TCRγ-positive, 1 case was TCRβ-positive, 2 cases were TCRδ-positive (two cases were positive for TCRγ and TCRδ). A subset of patients was evaluated for clonal TCR-Vβ expression by multiparameter flow cytometric immunophenotyping (IOTest Beta Mark TCR Vβ Repertoire Kit, Beckman Coulter, Miami, FL). Cases selected for comparison included a hepatosplenic gamma delta T-cell lymphoma, T-ALL, T-cell lymphoproliferative disorders (LPD), NOS, and a B-cell LPD. Flow cytometric analyses showed aberrations in normal T-cell antigen expression, including loss and co-expression of T-cell antigens. Molecular genetic analyses were performed routinely on all patients with questionable or documented T-cell LPD. Using patient specimens (peripheral blood, bone marrow aspirate, and lymph node cellular suspensions), the IOTest Beta Mark was incorporated in a 5-parameter, 4-color immunophenotyping strategy, with CD3 for selective gating and analyzed using a Beckman Coulter XL flow cytometer equipped with a 15 mW argon laser (excitation at 488 nm). Compensation was adjusted and data collected and analyzed with EXPO software (Beckman Coulter). Clonal proliferations were quantified using established criteria for the TCR-Vβ kit. Using an extensive algorithm of molecular genetic and multiparameter flow cytometric analyses, the diagnoses of clonal T-cell LPD can be

established. However, the TCR-Vβ repertoire analyses are an attractive and expedient alternative for complex T-cell disorders not easily accommodated by molecular techniques where prompt diagnoses of clonality are mandated for clinical management.

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ANTIGEN MAPPING: ANALYSIS TECHNIQUE FOR IDENTIFICATION AND CHARACTERIZATION OF MYELOPROLIFERATIVE AND MYELODYSPLASTIC DISORDERS.

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The traditional approach to clinical flow cytometric analysis in leukemia and lymphoma has focused on identification of individual cell populations by CD45 or forward light scatter by side light scatter display that are gated to generate phenotypic profiles on bivariate histogram displays. This method is, however, cumbersome and often poorly informative in many acute myelogenous leukemias, chronic myeloproliferative disorders and myelodysplastic syndromes (MDS). These disorders are characterized by abnormalities that involve precursor, differentiating and mature cell compartments and often involve multiple hematopoietic lineages. We have developed analysis protocols on the Coulter FC500 utilizing Coulter Cytomics software to generate color gated antigen maps which allow visualization of precursor and differentiating hematopoietic cell compartments on a single CD45 by side light scatter display. Two myeloid lineage antibody cocktails were evaluated containing the following antibody combinations: 1) CD45, CD13, CD15, CD33, CD34; and 2) CD45, CD11c, CD34, CD117, anti-Glycophorin A. The analysis protocol was designed to assign a specific color to each antigen and unique colors for significant antigen pair co-expression. Antigen (color) precedence was utilized to maximize resolution of precursor and differentiating cell compartments. This approach transforms the two parameter, CD45 by side light scatter histogram into a cell distribution display with six to nine antigen-defined cell populations visualized simultaneously. Specific antigen densities are viewed on appropriate bivariate antigen histograms without population gates so that all cell lineages, differentiation stages and cell inter-relationships can be assessed. The detail afforded by antigen mapping limits the number of histograms required for review and diagnosis of myeloproliferative and myelodysplastic processes. This analysis approach hinges on visualization of the hematopoietic compartment based on antigen-defined cell distributions, more closely reflects the biology of antigen expression within the hematopoietic compartment and capitalizes on the strength of flow cytometry as a diagnostic modality.

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CASE STUDY: FOUR-COLOR FLOW CYTOMETRY IDENTIFIES MAST CELL PRESENCE IN BONE MARROW SPECIMEN SUBMITTED FOR SUSPECTED SYSTEMIC MASTOCYTOSIS.

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We received bone marrow for flow cytometry from a 39 year old man suspected of having mast cell disease. Mast cells comprising 2% of the total cells analyzed were detected on our routine panel. Subsequent analysis revealed aberrant expression of CD25 on the mast cells, a feature supporting neoplasia. Normal mast cells are few in number in the bone marrow and commonly express CD117. It has been well documented that neoplastic mast cells often aberrantly express CD25 and occasionally CD2. Expression of CD25 serves as one of many criteria utilized in establishing a diagnosis of mastocytosis; however, it is not often performed in routine flow cytometry panels. We have found that even small numbers of increased mast cells can often be detected by routine display of forward light scatter versus CD117 for all gated cells in a single scatterplot, following a right angle light scatter versus CD45 gating strategy with color coding. Mast cells demonstrate increased granularity and moderate CD45. This places them in the mature granulocyte gate, which in the absence of mast cells will generally not contain high numbers of cells expressing bright CD117. Once the mast cells are suspected, appropriate combinations utilizing CD117, CD25, and CD2 can be performed to detect aberrant antigen expression. This case identifies the utility of displaying forward angle light scatter versus CD117 for all routinely gated (i.e. side light scatter versus CD45) populations in a single scatterplot and the importance of building a comprehensive flow cytometry antibody library to identify rare disorders.

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QUANTITATION OF CD2 EXPRESSION BY NORMAL AND NEOPLASTIC T CELLS IN PERIPHERAL BLOOD AND LYMPH NODE.

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CD2 is expressed on greater than 95% of thymocytes and peripheral T cells. CD2 plays a role in mediating T cell adhesion and facilitates antigen recognition in the thymus. Engagement of the CD2 antigen with its ligands induces T cell activation and proliferation. CD2 expression is modulated by various stimuli, including concanavalin A, anti-TCR beta, anti-CD3, IL-2, PMA/ionomycin and dexamethasone. The level of CD2 expression influences the intensity of TCR signaling in T cells, proliferative capacity, (IL)-2 production and the ability to undergo apoptosis. The QuantiBRITE system for fluorescence quantitation was utilized to quantitate levels of CD2

expression in normal and malignant T cells in concurrent peripheral blood and lymph node specimens from patients being screened for experimental monoclonal antibody therapy for T cell malignancy. Malignant T cells frequently demonstrated abnormal levels of CD2 expression compared to normal T cells in the same specimen. The abnormal levels of CD2 expression in malignant T cells may affect proliferative capacity. The levels of CD2 expression in normal T cells differed in lymph node compared to peripheral blood specimens collected concurrently from the same patients. Therefore the tissue site influences CD2 expression in normal T cells. Differing levels of T cell antigen expression may have implications in the flow cytometric quantitation of antigen expression for monoclonal antibody expression.

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EFFECT OF ACUTE HYPERTRIGLYCERIDEMIA ON THE BINDING OF SERUM COMPLEMENT TO INTRAVENOUSLY INJECTED ULTRASOUND CONTRAST AGENTS.

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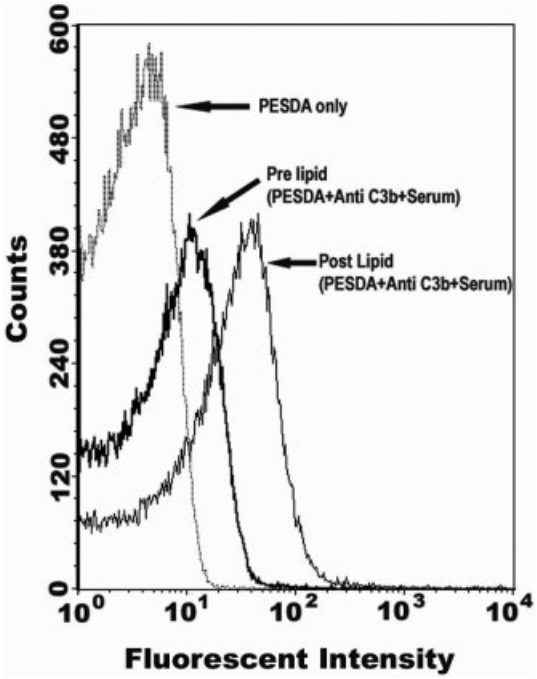
Background: Intravenous ultrasound contrast agents like albumin-encapsulated microbubbles normally pass freely through large and small vessels, but small numbers adhere to regions with endothelial dysfunction. In mice pre-treated with cobra-venom factor to selectively deplete complement, no adherence of microbubbles to the arterial endothelium has been detected, suggesting that complement-mediated binding is the possible mechanism underlying this phenomenon. Aim: To determine whether hypertriglyceridemia influences serum complement attachment to the surface of the contrast agent PESDA (Perfluorocarbon Exposed Sonicated Dextrose Albumin). Method: In three pigs hypertriglyceridemia was induced by intravenous infusion of intralipid 20%. The carotid arteries (CA) were imaged with a high-frequency ultrasound transducer equipped with low-mechanical index pulse sequence scheme following intravenous injections of microbubbles. PESDA (0.7x109 microbubbles) was combined with 0.5 milliliters of pig serum drawn before and after induction of hypertriglyceridemia, incubated at 37oC for five minutes, and washed. The serum-exposed microbubbles were then combined with C3b monoclonal antibody for 20 minutes, washed twice, and subsequently combined with R-phycoerythrin conjugated secondary antibody for 20 minutes. Microbubbles were analyzed on FACSCalibur to generate graphs of red fluorescent intensity. Results: Before intralipid, no adherence of microbubbles was detected in the CA by ultrasound. After induction of hypertriglyceridemia, adherence of PESDA was visually evident in all CA. Flow cytometry revealed complement binding to PESDA microbubbles in the presence of pig serum. This binding was increased during hypertriglyceridemia, expressed as a shift of mean fluorescent intensity curve to the right (Figure). Conclusion: In the setting of hypertriglyceridemia the increased binding of complement to the surface of PESDA microbubbles leads to adherence of these microbubbles to the arterial endothelium.



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LEUKAEMIA IMMUNOPHENOTYPING: AN APPRAISAL OF THE IMMUNOFLUORESCENCE PROGRAMME OVER THE LAST 10 YEARS FROM UK NEQAS FOR LEUCOCYTE IMMUNOPHENOTYPING.

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Leukaemias are a heterogeneous group of disorders, the management and treatment of which is diverse. It is essential to have an accurate diagnosis underpinned by precise and accurate laboratory data supported by internal and external quality control (QC) procedures. Here, we summarize the findings from the last 10 years of the UK NEQAS for Leucocyte Immunophenotyping (Immunofluorescence leukaemia) programme involving 213 participants from 25 countries. Blood obtained from consenting leukaemia patients is stabilized and 1ml issued to participants with an unstained blood smear for phenotypic interpretation. A panel of up to 12 antigens is requested for analysis, including 6 core antigens that are performance monitored, namely: CD3, CD5, CD7, CD13, CD19 and HLADR. Over the 10 years: mean inter-laboratory Co-efficient of Variation (CV) for scored antigens has fallen from 49% to 15%: with the mean percentage of laboratories 'out of consensus' falling from 16.8% to 3.1%: The percentage number of laboratories 'out of consensus' for diagnosis reduced from 4.9% to 2.4%. There has been an increase in the different fluorochromes in use from 2 to 9 with 3 colour flow cytometry used the most. This increase illustrates that participants are gaining experience and confidence in multi-

colour flow cytometry. It was found that the B-CLL's were the easiest for laboratories to phenotype and diagnose with 100% concordance, whilst the Plasma Cell Leukaemia's provided the greatest problems with only 50% laboratory concordance. The downward trend in CV's and percentage 'out of consensus' for both antigens and diagnoses, highlights the fact that EQA is essential. The advice and guidance provided, helps to promote awareness and knowledge, in turn increasing the quality of service for patients.

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STUDY OF THE IMMUNOPHENOTYPE,CYTOGENETIC ANOMALIES AND CLINICAL FEATURES IN 125 ADULTS WITH ACUTE MYELOID LEUKEMIA IN CHINA.

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Immunophenotyping is a widely used method for diagnosis and classification of acute myeloid leukemias (AML), and some immunophenotypes have been correlated with cytomorphological, cytogenetic and clinical features but their occurrence in AML differs in the various studies. In this study, 125 cases of acute myeloid leukemia at primary diagnosis with WHO classification were analyzed by high-resolution flow cytometry with a panel of 25 different monoclonal antibodies. The results were compared with the French-American-British (FAB) Cooperative Group classification, as well as with available cytogenetic data (93 cases) and clinical features on each case. The results demonstrated that CD33, CD38 and CD13 were the most commonly expressed antigen (95.1%,91.1% and 89.8%, respectively).CD7 was the most commonly expressed lymphoid antigen (20.4%), followed by CD19 (16.8%) and CD2 (15.2%). Some immunophenotypes correlated with FAB type, including increased frequency of CD2 in M3; lack of HLA-DR, CD34 and CD56 expression in M3; increased frequency of CD19 in M2, CD14 and CD56 in M5 and lack of MPO in M0, when compared with all other AMLs ($P < 0.05$). An abnormal karyotype was detected in 51 cases (54.8%). CD22, CD56 and TdT expression appeared significantly associated with chromosomal anomalies($P < 0.05$). Significant associations between immunophenotypic and karyotypic features, FAB subgroups were observed: all t (8; 21) leukemias demonstrated M2 morphology and high CD15, CD34 and CD56 expression. Lymphoid lineage antigens were not detected in M3 with t (15; 17). In addition, the expression of both CD7 and CD14 was significantly correlated with higher WBC count; CD4 or TdT expression with increased age; CD4, CD14 or CD56 expression with higher platelet count. No significant correlation was found between hemoglobin level and markers. The relationships of immunophenotypings and genetic features with treatment and prognosis are currently being studied. In conclusion, significant correlation between immunophenotypes and cytogenetic features in AML strongly suggested that the abnormal antigen expression is tightly linked with aberrant genetic changes. Analysis of high resolution flow cytometry with WHO classification is a powerful tool to be used in primary leukemia diagnosis as well as in study of understanding leukemia malignancy.

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ANALYSIS OF Bcr/Abl SIGNALING USING LARGE PANELS OF PHOSPHO-SENSITIVE ANTIBODIES.

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The Bcr/Abl fusion tyrosine kinase is constitutively active and is associated with CML and ALL. In this study, we analyzed cell signaling in a number of untreated and Gleevec-treated Bcr/Abl expressing cells lines (b3a2, b2a2, and e1a2 isforms) by flow cytometry using a broad panel of phospho-sensitive antibodies to determine the degree of signaling heterogeneity in model cells lines. Using this paradigm, we identified a number of phosphoproteins involved in the Ras/MAPK, PI3K/Akt, and Jak/Stat pathways, including c-Cbl, CrkL, Erk, FAK, Shc, SHP-2, and Stat5, that were constituatively activated in untreated cells and markedly decreased by Gleevec treatment. Interestingly, there were differences in the signaling profiles among the cell lines. These differences may represent differences among the Bcr/Abl signaling pathways and may have significance for disease treatment. These results demonstrate the use of phospho-specific antibodies as tools to analyze complex signaling in leukemic cells. This type of analysis may lead to the identification of signaling proteins that are robust biomarkers and/or potential therapeutic targets.

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THE CORRELATION DIMENSION CAN BE USED TO DETECT MONOTYPIC B-CELLS IN PERIPHERAL BLOOD: A NEW METHOD FOR FLOW CYTOMETRY DATA ANALYSIS.

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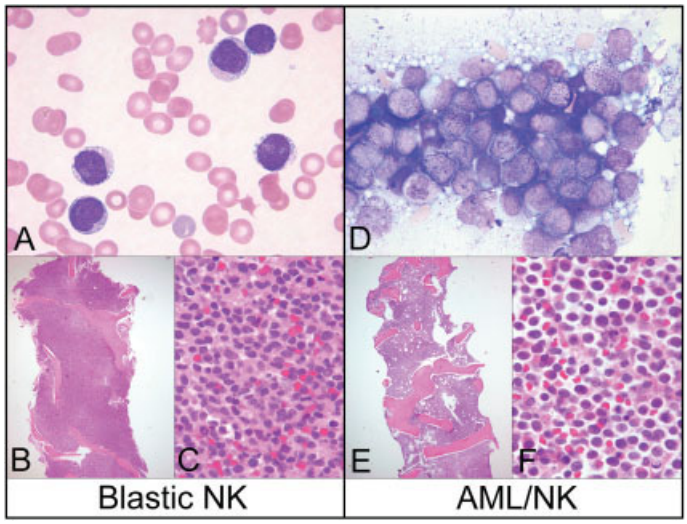
Chronic lymphocytic leukemia (CLL) exhibits decreased peripheral blood complexity as a neoplastic lymphocyte clone replaces the blood's normal cellular components. The reduced complexity manifests as altered distribution of the CD45-sidescatter and CD19-gated kappa-lambda immunoglobulin light chain expression data measured with a flow cytometer. The altered data distribution can be quantitated using the correlation dimension, a mathematical tool developed to measure the dimensionality of fractal objects. The correlation dimensions of peripheral blood CD45-sidescatter and kappa-lambda expression data for 23 non-neoplastic (negative) patients and 22 patients with CLL were compared. Comparison of the populations using Student's 2-tailed t test revealed a significant decrease in the correlation dimension of the CLL group for both CD45-sidescatter and CD19-gated kappa-lambda data (both p-values <0.001). For the CD45-sidescatter data the mean correlation dimensions of the negative group and the CLL group were 1.12, SD 0.00975 and 1.02, SD 0.00714, respectively. Analysis of CD19-gated kappa-lambda data revealed the mean correlation dimensions of the negative group and the CLL group were 0.882, SD 0.00708



Abstract #39: Figure-A, B&C: Blastic NK cells and Figure-D, E&F: AML/NK leukemic blasts

and 0.581, SD 0.00155, respectively. There was no overlap in correlation dimension between CLL and negative groups using CD19-gated data. The reduced correlation dimension reflects the decreased complexity of the peripheral blood flow cytometry data in CLL. This study demonstrates that the correlation dimension can, in principle, be applied to flow cytometry data. It may be a useful tool in quantitating data distribution in clinical flow cytometry.

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BLASTIC NATURAL KILLER (NK) CELL LEUKEMIA (AGRANULAR CD4+CD56+ HEMATODERMIC NEOPLASM)-A CASE REPORT.

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We report a case of CD4+CD56+ malignancy with a leukemic presentation and without skin lesions at the time of diagnosis. This was a 85 years old white male who presented to the clinic with chronic fatigue and found to have anemia and had high white count of 88,000/Ïl with 90% lymphoblastic-like cells. These cells were agranular with strong expression of CD4 and CD56 and absence of myeloid, pan-B or pan-T lymphoid markers by flow cytometric analysis. Immunoglobulin heavy chain and T cell receptor gamma subunit gene rearrangements of the cells by polymerase chain reaction (PCR) were both germline. Cytogenetic study showed multiple complex chromosomal abnormalities. The morphologic, immunophenotypical and molecular characteristics of the neoplastic hematolymphoid cells are consistent with blastic natural killer (NK) cell leukemia /or so-called agranular CD4+CD56+ hematodermic neoplasm, that was recently proposed. Recent updates on the disease with focus on the cell of origin are reviewed. In addition a case of myeloid /NK cell acute leukemia is presented here for comparison.

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FLUDARABINE RESISTANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA DETECTED BY A NOVEL IN VITRO MULTIPARAMETER FLOW CYTOMETRIC CYTOTOXICITY ASSAY.

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Fludarabine is a first line drug for B-cell chronic lymphocytic leukemia. However, about 10 to 20% of patients do not respond to Fludarbine therapy, associated with a poor prognosis. Detection of such drug resistance prior therapy not only predict prognosis, but also prevent patients from receiving expensive and ineffective chemotherapy. To develop an in vitro drug resistance assay for routine clinical samples containing leukemic cells admixed with normal hematopoietic cells, we applied multiparameter flow cytometry to measure specific drug-induced cytotoxicity on leukemic cells after they were incubated in cultures containing fludarabine. Leukemic cells were identified with antibodies against CD5 and CD19. Cytotoxicity was assessed with Annexin V and 7-AAD. Leukemic cell survival indexes were calculated by expressing viable leukemic cells in test cultures in percent of viable leukemic cells in control cultures. Fludarbine induced time- and dose-dependent apoptosis in leukemic cells. Of 35 samples tested, leukemic cell survival indexes ranged from 13 to 100% at the end of two-day incubation period with Fludarine at 2.5 μg/ml. Of five most resistant samples, four were found to have p53 deletion by FISH and one was cytogenetically normal. There was no consistent association between leukemic cell survival index and other cytogenetic abnormalities including trisomy 12, deletion of ATM, and deletion of RB1. The association between extreme drug resistance and p53 deletion validates the predicative value of the assay for clinical response since patients with p53 deletion almost invariably have a poor clinical response to fludarbine as previously reported. Therefore, in vitro multiparameter flow cytometric cytotoxicity assay can be performed on routine clinical specimens to identify the subsets of patients who will not benefit from Fludarabine therapy.