Belgian Society for Analytical Cytology symposium

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A summary of the BVAC/ABCA program hosted by the general annual meeting of the BHS in Ghent on Friday, January 31st, 2014.

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Introduction

IFor the second time, during the course of its general annual meeting, the BHS hosted a satellite meeting organised by the Belgian Society for Analytical Cytology (BVAC/ABCA) to bring together clinicians with laboratory scientists and staff. The BVAC/ABCA program included lectures in various fields of flow cytometry and analytical cytology by renowned national and foreign speakers. All the presentations are available on the BVAC/ABCA website: http://www.cytometry.be/Gent2014.html.

Accreditation of flow cytometry in Europe

Claude Lambert, Saint-Etienne, France

In Belgium and most European countries, accreditation of clinical flow cytometry is not yet mandatory. In France, all clinical laboratories will have to implement the ISO 15189 standard by November 1st 2020 for 100% of the tests performed, thus including flow cytometry (FCM).

Following the usual Ishikawa scheme, all components of the analytical process must be carefully controlled, i.e. metrology, instruments, procedures, operators, sample quality and environmental conditions. Claude Lambert dedicated most of his lecture to operator qualification and procedure validation.

The ability of technicians to perform FCM-based testing is critical to ensure overall quality. FCM is a complex technology, usually not included in the basic training of laboratory technicians, and involving delicate manual procedures, instruments and dedicated software's. A specific education on the theoretical and practical aspects of FCM must be provided beforehand to all personnel involved. Close supervision is mandatory until sufficient experience has been acquired and before autonomy may be granted. Field evaluation, questionnaires and an interview with the supervising biologist must be conducted to qualify lab technicians. Continuous education must be regularly followed, through internal or external workshops. Belgian and international scientific societies are active in organising continuous education programs for technicians and scientists.

Procedure validation in FCM has been hotly debated in international forums.[1,2] Validation schemes are generally available for standard clinical chemistry quantitative tests and include experimental determination of sensitivity/specificity, precision, bias, linearity, limits of detection and quantification, sample-to-sample contamination, analytical risk assessment and reference ranges. The applicability of such validation schemes to FCM has been challenged by many experts. Only validation of quantitative assays, i.e. enumeration of phenotypically-defined cell subsets such as T, B and NK cells or CD34+ cells for example, may be amenable, to some extent, to the validation criteria cited above. Leukaemia/lymphoma phenotyping are qualitative assays for which the limited availability and stability of cell samples, the lack of gold standard or external quality

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control programs, and the necessity for protocol adaptability, all prevent the application of usual validation schemes. Therefore, procedure validation in this case must focus on the control of instrument settings, targets of fluorescence intensity and optical compensations. Procedures must detail decision thresholds, phenotype definitions, gating strategy, data analysis and reporting.

Flow cytometric analysis of cerebrospinal fluid

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Clinical parameters used to define risks of central nervous system (CNS) involvement in B-NHL are of limited value. Most patients who develop clinical CNS disease have a negative CNS cytology at initial staging, thus alternative flow cytometric (FCM) methods of lymphoma cell detection were developed. In a series of 51 newly diagnosed cases of aggressive NHL, Hegde et al. reported increased sensibility of FCM over conventional cytology (CC) to detect malignant cells in the cerebrospinal fluid (CSF; eleven FCM positive cases versus one CC positive).[3]

FCM analysis of CSF has several pitfalls: limited cell viability, low volume and cell numbers, peripheral blood contamination, etc. Several reports have demonstrated that cell viability decreases rapidly, with a 50% decay as soon as 30 minutes after the spinal tap. It is therefore recommended to directly stabilise the CSF in a fixative agent (Transfix®) or culture medium with foetal bovine serum prior to the transport to the laboratory. Next, in order to make the best use of the small amount of material available, it is advised to skip the cytological analysis completely and to phenotype and count cells simultaneously by FCM with a single platform approach using calibrated latex beads.[4] Phenotyping of lymphocyte subsets and detection of malignant cells may be carried out using the 'small sample tube' (SST), a single 11-marker 8-colour combination tube standardised by the EuroFlow Consortium.[5] Peripheral blood contamination of the CSF is detected by the presence of red blood cells and an increase in the proportion of granulocytes (which should represent only 7% of the leucocytes in normal CSF).

What is the clinical impact of occult leptomeningeal disease (FCM+ CC-)? In a multicentric study to be published shortly, the increased sensitivity of FCM over cytology to detect CNS infiltration in diffuse large B cell lymphoma (DLBCL) and Burkitt's lymphoma (BL) was confirmed.[6] Furthermore, occult CNS disease was associated with shorter survival and increased rate of CNS relapse in DLBCL, while there was no difference among patients with a positive compared to negative CNS cytology. In BL, occult CNS disease was associated with a higher rate of CNS relapse, but not with shorter survival. This study suggests that occult CNS involvement is a clinically meaningful finding.

Finally, the significance of soluble B-cell derived proteins in the CSF was discussed. A promising marker appears to be soluble CD19 (sCD19) which was recently reported to be significantly associated with the presence of neurological symptoms. The combination of sCD19 CSF levels and FCM improved the detection of CNS disease and increased sCD19 was associated with poorer event-free and overall survival in DLBCL and BL.[7]

Quantification of plasma cells by flow cytometry: methodology and applications

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Quantification and characterisation of plasma cells by flow cytometry (FCM) requires a rigorous pre-analytical procedure. A two to five ml bone marrow sample should be collected, mixed with heparin or ACD, kept at room temperature and analysed within 48 hours. Cell aggregates must be disrupted with a 21G needle. Ammonium chloride is recommended as red blood cell lysing reagent but contact with the bone marrow cells should be kept as short as possible. Even when these recommendations are followed, the percentage of plasmocytes quantified by FCM is usually lower than by conventional cytology. Contamination by peripheral blood, incomplete disruption of cell aggregates, cell loss during centrifugations and RBC lysing procedures may contribute to the underestimation of plasmocytes by FCM. Quantification of plasmocytes relative to CD45dim CD117+ CD34+ myeloid precursors has been proposed to circumvent this issue.[8]

FCM is used to distinguish malignant and normal plasmocytes. The clonal nature of malignant plasmocytes may be demonstrated by the monotypic expression of immunoglobulin (Ig) kappa or lambda light chains. As plasmocytes generally lack surface Ig, labelling should target cytoplasmic light chains after cell permeabilization. Aberrant expression of surface antigens may also be used to indicate the neoplastic origin of plasmocytes. On a CD45dim, CD38+CD138+ backbone, the expression of CD56, CD28 and CD117, or the absence of CD19

and CD27, are all suggestive of neoplastic plasmocytes. As no single marker is diagnostic of clonal plasmocytes, composite phenotypes must always be used.[9] Although downregulation of CD45 seems to be related to shorter survival, the prognostic significance of specific plasma cell phenotypes remains to be determined.

In monoclonal gammopathy of undetermined significance (MGUS), FCM analysis of plasma cells is relevant to estimate the risk of progression to multiple myeloma (MM). In symptomatic MM, the ratio of clonal plasmocytes within the bone marrow plasma cell compartment has prognostic significance: patients with less than 95% clonal plasmocytes have longer PFS and OS.[10] MM patients with a 'MGUS-like signature' have an excellent outcome, irrespective of the depth of response to therapy.[11]

Plasma cell quantification by FCM may be used to assess minimal residual disease and response to therapy. Although less sensitive than molecular biology; FCM is fast, relatively inexpensive and applicable to most patients. The detection limit is 1% to 0.01% malignant plasmocytes, depending on the number of phenotypic aberrancies. In patients undergoing autologous transplant, MRD assessment by FCM is predictive of outcome, and demonstrable in patients with immunofixation-negative complete response, as well as in patients with favourable and unfavourable cytogenetics.[12] A recent report has highlighted a major heterogeneity in the MRD analysis procedure among US laboratories. Efforts in standardisation of antigen panels, number of cells tested, sample enrichment, cell concentration and time of bone marrow aspiration remain to be undertaken.

The basophil activation test: state of the art

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Type I immune pathologic reactions are mediated by IgE and directed against foreign antigens, i.e. allergens. The symptoms are elicited by binding of allergens to IgE absorbed on the surface of basophils and mastocytes, followed by the release of histamine, leukotrienes and other mediators. Specific IgE (sIgE) are produced by B lymphocytes switched to IgE by primed Th lymphocytes, which are stimulated by the presentation of the allergen by dendritic cells. Allergens include airborne allergens, food, drugs, venoms and latex. Allergy is diagnosed by history and physical examination, tryptase assay, quantification of IgE and determination of sIgE by component-resolved diagnosis using microarrays, skin, and provocation tests. None of these tests have absolute sensitivity.

The basophil activation test (BAT) is based on the ex vivo stimulation of basophils in whole blood by a suspected allergen. Using flow cytometry, basophils are identified among leucocytes using anti-IgE or CRTH2 and responding basophils are defined by the upregulation of CD63 or CD203c. A new type of BAT, developed by Ebo et al, is based on the staining of basophils by fluorescent diamine oxidase (DAO) which binds to histamine. Upon basophil stimulation, histamine is released and the signal generated by DAO decreases (HistaFlow®). BAT tests may use the patient's basophils, or in the indirect version, donor basophils incubated with the patient's serum. BAT has primarily been used to test for allergy to neuromuscular blocking agents, β-lactam antibiotics and wasp venoms. The specificity is usually very high, with a sensitivity of 50-80% depending on the nature of the allergen.[13]

Rocuronium is a curarising myorelaxant, potentially responsible for life-threatening allergy during anaesthesia induction. As re-exposure to the agent is out of the question, provocation tests cannot be performed and skin testing or specific IgE assays are of limited use since they cross-react with safe neuromuscular blocking agent (NMBA) analogs. Thus, BAT with rocuronium is a promising alternative test, with an estimated sensitivity of 80% and a specificity of 96%. Complementary to skin testing, BAT may confirm the patient's reactivity, identify cross-reacting NMBA analogs (BAT+) and tailor safe alternatives (BAT-). Furthermore, BAT has a better predictive value of the clinical outcome than sIgE testing.

Another interesting application of BAT is hypersensitivity to opiates. The differential diagnosis of opiate allergy and non-specific histamine release is difficult. Skin testing is unreliable and sIgE assays are unavailable. Using BAT, reactivity to pholcodine, codeine and morphine may be resolved, allowing the identification of the culprit allergen and the tailoring of safe alternatives.[14] BAT is also applicable to plant and fruit allergies. Interestingly, recent data pointed out that cannabis users may be sensitised to lipid transfer proteins, present in cannabis sativa, and known as important allergens in various plant and fruit allergies, such as peach and kiwi.[15] Finally, BAT may be used in the diagnosis of auto-immune urticaria, using the indirect method. In this case, donor basophils are incubated with the patient's

serum and may respond to autoantibodies reacting with IgE or IgE receptors on the basophil membrane, FceRI.

Circulating tumour cells: biomarkers in oncology

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In 2005, Braun et al demonstrated that in breast cancer patients, the presence of micro metastases in the bone marrow were associated with shorter survival.[16] To avoid repeated bone marrow sampling, methods to detect epithelial cancer cells in peripheral blood were developed. These included mainly immunocytochemistry and RT-PCR. The CellSearch® system is based on immunocapture of EpCAM+ cells, followed by immunocytochemistry of cytokeratin+ CD45- nucleated cells. Though this system has been cleared by the FDA for the progression assessment of metastatic breast, prostate or colorectal cancer patients, to date it has not been recommended as a standard of care by the scientific societies. RT-PCR of epithelial transcripts is more sensitive than immunocapture techniques and predicts prognosis in early stage breast and colon cancers as well as in metastatic prostate cancer.[17]

Problems that face CTC detection are its poor sensitivity and specificity. The efficiency of CTC immunocapture may not be optimal, thus leading to an underestimation of the CTC's proportion. Larger blood volume processing may be carried out by leukapheresis and enrichment may be optimised using size dependent filtration or immunocapture with other targets such as CXCR4, CD44 and EGFR. All circulating epithelial cells may not be malignant cells, consequently further characterisation is mandatory. Cells which initiate micro metastases in breast cancer have phenotypic features of cancer stem cells (CD44+CD24-AlDH+) while macro metastases have a profile similar to the primary tumour. CTC may also present epithelial-to-mesenchymal transition (EMT), with downregulation of EpCAM, expression of vimentin, N-cadherin and TWIST, and increase in metastatic capacity. Overall, the improvement of accurate CTC detection may be achieved by RT-PCR assessment of stem cell markers and EMT.

The prognostic and predictive relevance of CTC has been shown in many settings: in metastatic breast cancer, urinary bladder cancer, hepatocellular carcinoma and squamous cell carcinoma of the oral cavity, CTC are associated with relapse and/or shorter survival. Phenotypic characterisation of CTC for the expression of therapeutically relevant targets has been shown in breast (HER-2, ER), colon (EGFR) and prostate (ERG) cancer. The next question to address is whether the CTC assay can guide therapeutic decisions. DETECT III is a multi-centre randomised phase II trial that studies the efficacy of lapatinib, a tyrosine kinase inhibitor, in patients with initial HER2-negative metastatic breast cancer and HER2-positive CTC.[18]

Finally, in xenograft assays, the functional capacity of breast cancer CTC to initiate metastases has been recently reported. In a small cohort of patients with metastases, the number of EPCAM(+)CD44(+)CD47(+) MET(+) CTCs, but not of bulk EPCAM(+) CTCs, correlated with lower ov erall survival and increased number of metastatic sites.[19]

Monoclonal B cell lymphocytosis

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With the generalisation of flow cytometry analysis of patients with lymphocytosis, it has become apparent that many otherwise healthy individuals harbour small B cell clones. Monoclonal B cell lymphocytosis (MBL) indicate the presence of monoclonal B lymphocytes below a threshold of 5000 cells/μl, in the absence of any feature indicative of a B lymphoproliferative disorder. The stability of the B cell clone should be demonstrated by repeated assessment over a three month period. Approximately 1% of MBL cases develop progressive CLL per year. MBL is subclassified according to the phenotype of the monoclonal B cells. CLL-type MBL represents the vast majority of cases, with a Matutes-Catovsky score of four to five. Other cases may have a phenotype of atypical CLL, or of another lymphoproliferative disorder, such as mantle cell lymphoma, marginal zone lymphoma or lymphoplasmacytic lymphoma.[20] The workup of non CLL-like MBL requires cytogenetic analysis, CT staging and bone marrow analysis, and a follow up every three months.

On the other hand, CLL-like MBL only requires blood cell count and flow cytometry analysis every twelve months. CD10+ monoclonal B cells, even in small amounts, are not detected in the general population and should always lead to a comprehensive lymphoma workup.

Estimations of MBL incidence in the general population are largely dependent on the sensitivity of the flow cytometry protocol. With a 2-colour analysis, <1% of

individuals over 60 years old show an MBL clone, whereas an 8-colour protocol reveals small B cell clones in 10-20% of adults, the incidence increasing with age. In population studies reporting a high prevalence of MBL, the median number of CLL-like B cells ranges from one to ten cells/μl.[21] The term 'CLL-like B cells' is proposed for minimal levels of clonal B cells detected in population studies, while 'CLL-type MBL' should be used for cases with an absolute clonal B cell lymphocytosis >500 cells/μl. Individuals with CLL-like B cells show no evidence of progression, no reduction in overall survival and no increase in infection risk. In contrast, CLL-type MBL is associated with a 20% reduction in ten-year overall survival, and increased risk of severe infection, related to depletion of normal B cells and immune suppression.[22]

Other biological differences have been reported to distinguish the two entities. The majority of CLL-type MBL displays mutated IGVH, with a repertoire similar to CLL. A mutated IGVH repertoire is also observed in cases of CLL-like B cells, although different to CLL. Stereotyped BCRs are infrequent in CLL-like B cells in contrast to CLL-type MBL and Rai stage 0 CLL.[23] Therefore, CLL-like B cells, in contrast to CLL-type MBL, may not represent a pre-leukaemic state.

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