BELGIAN CONSENSUS RECOMMENDATIONS FOR FLOW CYTOMETRIC IMMUNOPHENOTYPING.

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1. INTRODUCTION

A vast amount of literature data exists on flow cytometric immunophenotyping of haematological malignancies: for this the reader is referred to some excellent international reviews (1,2,3,4,5,6). Other powerful clinical applications of flow cytometry are hematopoietic progenitor cell counting, diagnosis and follow-up of AIDS, diagnosis of primary immunodeficiencies (7), evaluation of platelet glycoproteins and function (8) and HLA-B27 (9,10,11,12) analysis and cell cycle analysis (CCA) with DNA-ploidy assessment. As a result of the implementation of these applications in the clinical laboratory and because of the large variation in the practical realisation a number of authors have stressed the need for proper quality control, guidelines, and consensus recommendations for each of the respective fields, i.e. immunophenotyping leukaemias and lymphomas (13,14,15,16,17,18), progenitor counting (19,20,21,22,23,24) AIDS follow-up (25,26) and DNA-cytometry (27,28,29,30,31,32,33).

The Belgian Association for Cytometry (BVC/ABC: Belgische Vereniging voor Cytometrie/Association Belge de Cytométrie) has recently organised a number of discussion forums (see acknowledgements). These forums served to extract the essential information from the above-mentioned literature of use in the specific Belgian context. The summary of these discussions can be found in this paper. The aim is to provide the prescribing physician and interested "layman" with a state of the art overview that highlights the different possibilities of flow cytometric evaluation. It also provides the Belgian cytometric laboratories with common testing panels, so that they can speak the same "language". In so doing, we hope to improve or gain consistent quality in Belgian cytometric laboratories.

The forum participants very carefully examined the informative content of every given marker within every given pathology. Most emphasis has thus been put on the selection of markers while other aspects, such as technical and sample handling, standardisation and validation of procedures, and the manner by which the information is generated and reported (34), are only briefly mentioned in this report. Recurrent issues however are mentioned in the general preceding remarks section. In a second part, we present the consensus recommendations on the immunophenotypic analysis of haematological neoplasia by flow cytometry. Progenitor cell counting and flow cytometric analysis in HIV will be discussed in separate chapters. The other applications fall beyond the scope of this article because they are so specialised (platelets), represent such rare pathologies (primary immunodeficiencies), are straightforward (HLA-B27) or are not based on immunophenotyping (CCA): for this the reader is referred to the international literature.

2. GENERAL PRECEDING REMARKS

Fresh, viable anticoagulated samples (35, 36) should be at the laboratory's disposal, together with all available clinical and diagnostic information pertinent to it.

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Solid tissue samples should be transported in sterile physiological solution to prevent dehydration.

This clinical information is essential since it will guide the laboratory professional to decide on the antibody selection. Apart from a minimal consensus screening set, additional more specific set(s) of markers will be presented for further discrimination of the minority of cases that may not be properly resolved by the first set. We leave it to the appreciation of every laboratory professional to decide whether or not one immediately uses a comprehensive panel or one uses the consecutive approach. In essence, it depends on a timesaving or reagent-saving attitude: both however are cost saving.

Data reporting of immunophenotyping differs from that of many other diagnostic tests in that it requires a lot of data interpretation. Careful evaluation and follow-up of the relative or absolute amount of the different lymphocyte subsets may be of importance in immune status evaluation. This is much less relevant in the approach to the diagnosis of haematological malignancies. The qualitative information about the combined presence or absence of different surface antigens and the cell surface density at which these are expressed is much more important than the quantitative information on the percentage of cells carrying a single given marker. Thus, although this quantitative information is present it is the qualitative information that should be highlighted on the physician's protocol. A descriptive summary of the phenotype of the abnormal cells -if present-should always be included, together with an estimation of its abundance. Since reporting percentages for each marker used is usually unnecessary or irrelevant, it is implicit that the inclusion of normal reference ranges in the report is inappropriate. Due to inevitable variable amount of peripheral blood contamination, it is even impossible to reliably propose normal range values for single surface antigen presence (37) in bone marrow.

It is important to note that apart from the markers relevant to the suspected malignancy, some additional markers are warranted as internal consistency checks. These may be different depending on the nature of the sample (peripheral blood, bone marrow, lymph node...) or of the cell cluster under study. An internal consistency checklist is presented in table 1.

Finally it should be stressed that the CD designation (CD = cluster of differentiation) is coined for the grouping of antibodies that exhibit specificity for the same given antigen, coined CD molecule/CD protein/CD antigen. In what follows the term CD may be used as short for either CD antibody or CD molecule. One should realise however that antibodies assigned the same CD number, thus recognising the same CD antigen, might exhibit different antigenic specificity since they can recognise different epitopes of the antigen. To facilitate comprehension we have included table 2 summarising the phenotypes detected by the CD clusters of antibodies that are relevant in what follows.

3. HEMATOLOGIC NEOPLASIA

There was a consensus on the importance of morphologic evaluation. Integration of morphologic and immunophenotypic data is critical in achieving diagnostic accuracy and minimising interobserver interpretative discrepancies (38).

Within every broadly defined cellular lineage (lymphoid, myeloid) and/or maturation stage (early, late) we will propose a minimal consensus set of markers to be evaluated in order to cover and delineate the majority of the relevant malignancies.

It is the opinion of the forum participants that much attention should be given in looking for the appearance of aberrant combinations of antigens that discriminate the patient's malignant cells from the normal counterparts. This patient specific information may then be useful in the detection of minimal residual disease after-

Table 1 — Consistency checks

nature of sample/cell	antibody cluster(s)	phenotypes/relevance
solid tissues, BM	CD45	Check on leukocytic origin, RBC contamination, blast discrimination*
normal lymphocytes	CD14	Check on monocyte contamination of lymphocytes
normal lymphocytes	CD16 or CD56	Check on peripheral lymphocyte sum: T+B+NK~ 100 §

* See reference,(69) § T-cell marker will always be found in the first set of every diagnostic panel.

Table 2 — clusters of antibodies discussed in the text

CD number	antigen	expression
CD1a	T6	Thy, LHC, DC
CD2	T11, Tp50, sheep RBC receptor	T, NK
CD3	CD3 complex	T
CD4	T4, HIV receptor	Th/i, M (low density)
CD5	Tp67	T, Bsub
CD7	Tp41, Fc receptor for IgM?	Tsub, some AML
CD8	T8	Ts/c, NKsub
CD9	p24	pre-B, M, Plt, Eo
CD10	neutral endopeptidase, gp100, CALLA	Lymphoid Prog, cALL, GCB, G
CD11c	α chain of CR4, gp150/95	M, G, NK, Bsub, B
CD13	aminopeptidase N, gp150	M, G
CD14	gp55, LPS receptor	M
CD15	Lewis^x, 3-FAL, X-hapten	G
CD16	Fcγ-receptor type IIIa, gp50-65	NK, G, Mac
CD19	Bgp95	B and B-prog
CD22	Bgp135	surface Bsub, cyt. pan B
CD23	FceRII, gp50-45, Blast-2	Bsub
CD25	IL-2Rα, Tac	T act, B act, M act
CD33	My9	M, normal and malignant myeloid prog
CD34	My10	prog, EC
CD36	Platelet GPIV, GPIIIb, OKM-5 antigen	M, Plt, (B)
CD38	T10, gp45	PC, Thy, Tact, precursors
CD41	GPIIb/IIIa complex	Megakaryocytes, Plt
CD42b	GPIb-α	Megakaryocytes, Plt
CD43	Leukosialin, sialophorin, gp95-115	T, G, M, NK, Plt
CD45	T200, leukocyte common antigen (LCA)	Leukocytes
CD45RO	restricted T200, gp180	Tsub, G, M
CD55	decay-accelerating factor (DAF)	broad, absent in PNH patients
CD56	NKH1, isoform of N-CAM	NK, lymphocytes act
CD57	HNK1, gp110	NK, T, Bsub, brain
CD58	LFA-3	Leukocytes, epithelial
CD59	gp18-20, HRF-20, protectin, Ly6 analogous	broad
CD61	GPIIIa, Integrin β3 chain	Plt
CD64	FcγRI, high affinity Fc-IgG receptor	M, G act
CD66b	CD67 (now obsolete CD), p100, CGM6, NCA95	G
CD68	gp110, macrosialin	M, Mac (mainly cyt.)
CD71	transferrin receptor, T9 antigen	T and B act, Mac, proliferating cells
CD79α	mb-1, Igα	B
CD79β	B29, Igβ	B
CD103	α-chain of HML1, αEβ7 integrin, integrin αE	Intestinal IEL, hairy cells
CD117	stem cell factor receptor, steel factor receptor, c-kit	Mast cells, myeloid prog
CD138	syndecan-1	plasma cells, pre-B (weak), epithelial

Abbreviations: act = activated, B = B cells, cALL = common acute lymphoblastic leukaemia, DC = dendritic cells, EC = endothelial cells, Eo = eosinphils, G = granulocytes, GCB = germinal center B cells, IEL = intraepithelial lymphocytes, LHC: Langerhans cells, M = monocytes, Mac = macrophages, NK = natural killer cells, PC = plasma cells, Plt= platelets, prog = progenitor cells, sub = subpopulation, T = T cells, Thy = thymocytes.

wards. In most cases a marker present throughout the complete lineage will be necessary to gate out the relevant (suspected malignant) cell subset. Other surface antigens can further be evaluated within this restricted population. Simultaneous triple or quadruple labelling is strongly recommended for this approach. It saves reagents (avoiding duplicate usage of the lineage marker) and cells and it is the only way to directly document multiple marker coexpression at the single cell level. The workshop committee sees no further obstacles in advocating this approach: the optical bench of all modern analytical machines perfectly permits the separate collection of at least three fluorescence signals and the monoclonal antibody market offers a sufficient number of appropriately conjugated reagents.

3.1 Mature lymphoid cell malignancies: Chronic lymphoproliferative disorders + non-Hodgkin lymphomas

The first set (see table 3) of reagents serves to determine the origin (T- or B-cell) of the lymphoid neoplasm. Since the majority of the lymphoid neoplasm's are of B-cell origin this will practically always be followed by a second set of reagents that is needed to evaluate the «Catovsky» score (39) for the B-CLL phenotype. The immunophenotype typical for a B-CLL comprises five major parameters: expression of CD5 and CD23, weak expression of sIg and CD79b (40) and absence of FMC7 reactivity. To restrict the evaluation to the B-cell compartment, the committee suggests the use of CD19 or CD20 as gating tool.

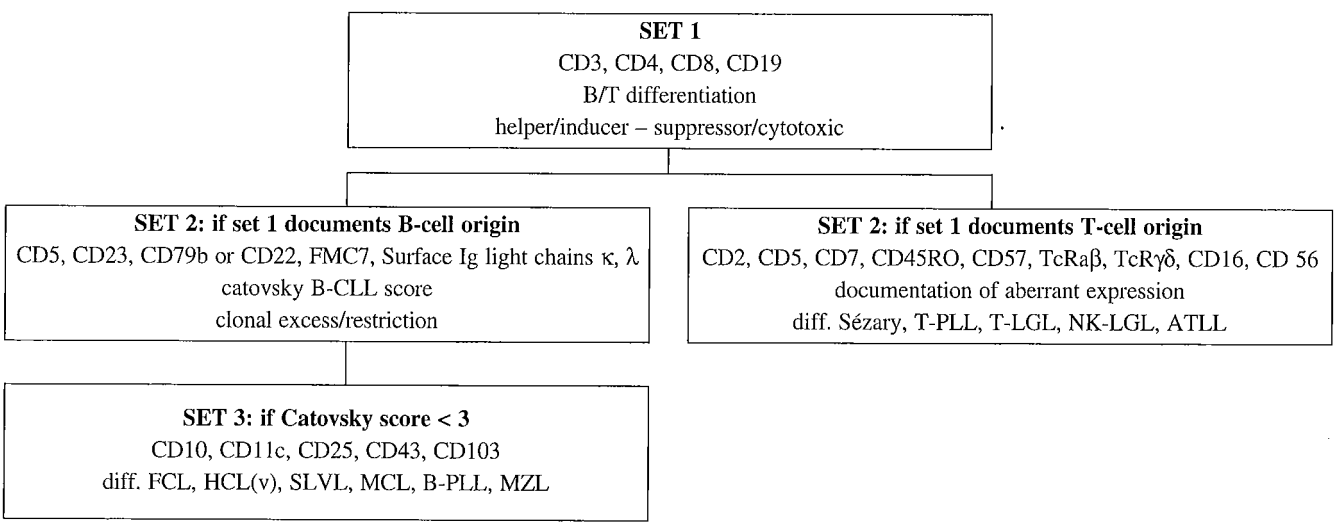
A third additional set serves to confirm the remaining B-cell pathologies (41,42,43) based on the fact that some markers can be associated to certain pathological cell types. Follicular centre lymphoma cells are positive for CD10, hairy cell leukaemia cells and its variant form are positive for CD103 and splenic lymphoma with villous lymphocytes are mostly negative for CD103 and CD25 but positive for CD11c. Finally, mantle cell lymphoma cells are commonly negative for CD10 and CD23 but positive for CD5.

If the first set reveals T-cell origin than a second set concentrating on additional pan T-cell markers is warranted because many leukaemic T-cell populations express an aberrant combination compared to normal T-cells. The true clonal origin of a T-cell population can further be explored by evaluating single Vb protein expression using an exhaustive Vb antibody panel (44) but can only be resolved however by tracing the rearrangement of the T-cell receptor at the DNA (45,46) or RNA (47,48)-molecular level, albeit for the latter not with 100% accuracy.

3.2 Plasma cell dyscrasias

Plasma cells have traditionally been difficult to assess by flow cytometry, possibly due to their great

Table 3 — chronic lymphoproliferative disorders: algorithm



Abbreviations: CLL = chronic lymphocytic leukaemia, SLL = small cell lymphocytic lymphoma, FCL = follicular center cell lymphoma, HCL (v) = hairy cell leukaemia (variant), SLVL = splenic lymphoma with villous lymphocytes, MCL = mantle cell lymphoma, PLL = prolymphocytic lymphoma, MZL = marginal zone lymphoma, LGL = large granular lymphocyte, ATLL = adult T-cell lymphoma/leukaemia

Table 4 — plasma cell malignancies

panel	antibody cluster(s)	phenotypes / relevance
SET 1	CD45, CD38(high density), CD138	Plasma cells / myeloma cells (CD56+)
SET 2	Cytoplasmic κ, λ	Clonal restriction → myeloma cells

fragility and selective loss during density gradient separation methods. Fresh samples and immediate processing without density separation are prerequisites to obtain representative results: CD138 expression fades within hours after collection.

In current clinical practice slide preparations for cytoplasmic κ, λ are considered most useful to evaluate the clonal origin of plasma cells: the flow cytometric markers for plasma cells (see table 4, CD45 absence or dim expression and CD38 presence with high surface density or CD138+) do not discriminate between normal plasma cells and myeloma cells although co-expression of CD56 can be of help (49).

In Waldenström's macroglobulinaemia/lymphoplasmocytic lymphoma, diagnosis is especially based on morphology and other laboratory results. CD38 is not as bright as in myeloma and many B-lineage antigens are brighter than in CLL/SLL or myeloma. Clonality can be shown by surface light chain restriction.

3.3 Myelodysplastic Syndrome (MDS) and Chronic Myeloid Leukaemia (CML)

Morphological examination of these samples is necessary before considering flow cytometric evaluation: If less than 20% blasts are present typing is of little relevance unless for confirmation of the microscopical blast count. However the diagnosis of MDS is frequently established during the investigation of a persistent and unexplained leukopenia/neutropenia and as such the sample may need to be studied for excluding other pathologies such as lymphoma or HCL.

In myelodysplastic syndromes flow cytometry may have potential diagnostic utility: despite a low number of CD34+ cells it is possible to observe altered (CD13, CD33) or aberrant (CD56) antigen expression. Nevertheless, the clinical implications have not yet been established. High HLA-DR and low CD11b expression may predict evolution of the syndrome with early conversion to leukaemia (50).

Flow cytometric immunophenotyping is not recommended in the chronic phase of CML, but is useful in cases of a suspected blast crisis (CD34+ cells), in which case the sample must be further handled as described under "acute leukaemias" (see further). The expression of CD117 (c-kit) may be of use in some cases where the blast cells do not express CD34. It is important in these cases to distinguish lymphoid from myeloid blasts (see further Table 6) (51).

3.4 Paroxysmal Nocturnal Haemoglubinuria and aplastic anaemia

The loss of glycosylphosphatidylinositol (GPI)-anchored antigens is most often seen in RBC and granulocytes, followed by monocytes, and less often in lymphocytes. Since severely affected erythrocytes may be selectively lost, the workshop committee suggests to evaluate the presence or absence of these antigens on the surface of the relevant leukocyte populations: i.e.

Table 5 — PNH and AA

Panel	antibody cluster(s)	phenotypes / relevance
SET 1	CD14, CD64	GPI-anchored antigen CD14 on monocytes (CD64+)
	CD16*, CD15	GPI-anchored antigen CD16 on PMN (CD15+)
SET 2	CD55, CD58, CD59 or CD66b	GPI-anchored antigens on erythrocytes/granulocytes

* Use an ab that recognises a GPI-linked CD16 molecule

CD16 on granulocytes and CD14 on monocytes (see table 5). The relevant cells can be gated by their light scatter properties or alternatively by gating on a cell type specific marker such as CD64 (for monocytes) or CD15 (for granulocytes).

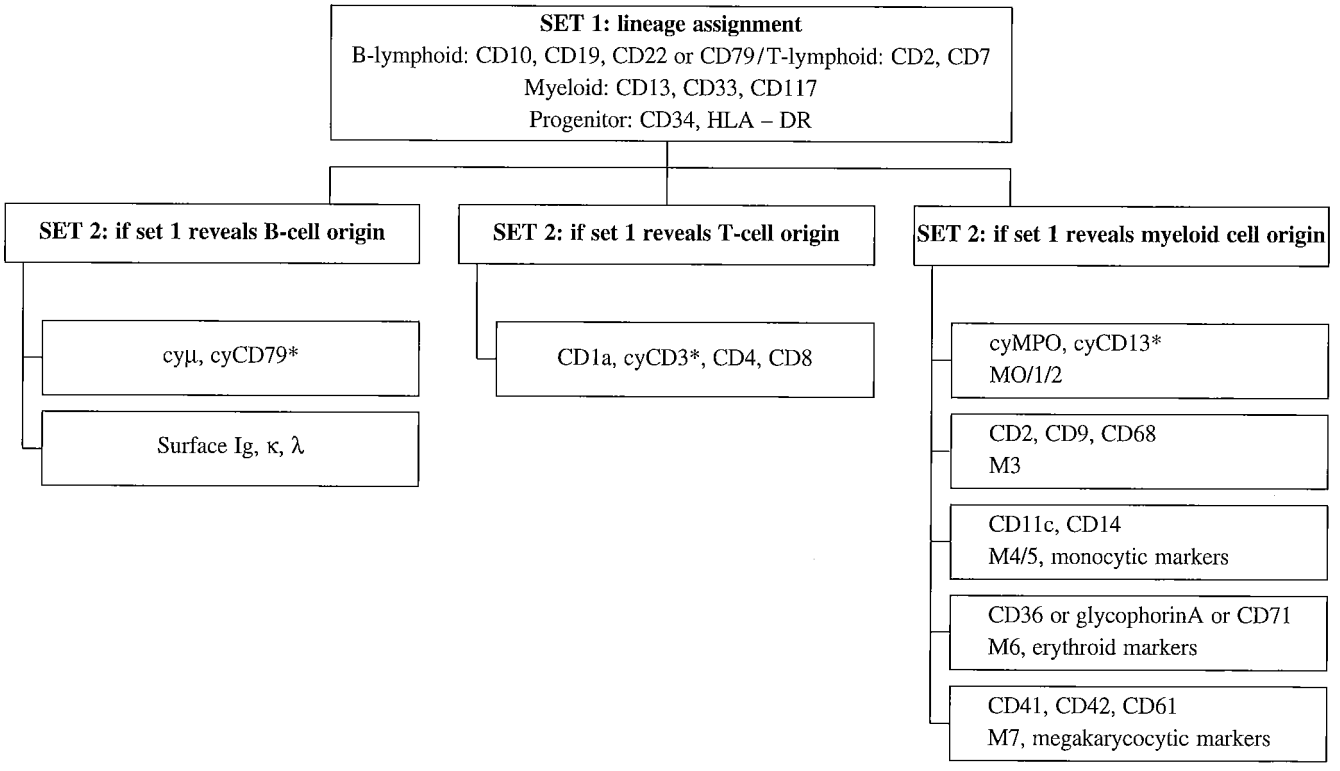
As a second set, GPI-anchored antigens on erythrocytes can be considered.

3.5 Acute Leukaemias

Upon diagnosis when the patient presents with a majority of blasts, a first set of markers serves to pinpoint the origin of these cells. This needs to be followed by a mandatory second set which is different depending on the results of the first set (see table 6). This set allows to establish a classification of ALL according to the degree of B-lymphoid or the degree of thymic differentiation. The workshop committee stresses the need for including cytoplasmic CD3, CD22 or CD79 and MPO since they are the most important and earliest lineage specific antigens for the T-, B- and myeloid lineage respectively: especially in cases where the use of set 1 doesn't provide any lineage clue the cytoplasmic markers become indispensable.

Table 7 enables to assign a lineage marker score based on the propositions of the European Group for the Immunological Characterisation of Leukaemias (EGIL) (52). A lineage score that equals or exceeds two, without any positivity for another lineage makes the lineage assignment definitive. All other cases (i.e. score 2 for 1 lineage but score 0.5 to 1 for another lineage or score <2 for one lineage with score 0 for all other lineages) warrants further examination to reduce the number of undetermined (score <1) or ambiguous (score 1 for several lineages) results. Lineage score (2 for more than one lineage could point to a biphenotypic leukaemia but one should be cautious for overinterpretation of weak non-specific binding. Because of the high phenotypic heterogeneity among acute leukaemias, defining new categories without some well-defined bio-

Table VI — Acute Leukaemias: algorithm



Abbreviations: * = If corresponding surface expression is absent.
M0 → M7 = Acute leukaemia classification according to the French-American-British (FAB) proposals: M0= minimally differentiated myeloid leukemia, M1 = myeloblastic without maturation, M2 = myeloblastic with maturation, M3 = promyelocytic, M4 = myelomonocytic, M5 = monocytic leukaemia, M6 = erythroblastic leukaemia and M7 = megakaryoblastic leukaemia.

Table 7 — lineage score

points	B lineage	T-lineage	Myeloid lineage
2	CD22* or CD79*	CD3*	MPO (enzymatic or antigenic)
1	CD19	CD2	CD13
			CD33
			CD117
0.5	CD10	CD7	

Abbreviations: * = Surface or cytoplamic presence, MPO = myeloperoxidase

logical or clinical rationale seems unwarranted. It is preferred to classify a leukaemia according to its main lineage. If not possible, it is recommended to describe the distinctive unusual features, and apply the non-judgmental term "unclassifiable acute leukaemia" (5, 18). However, it should be stressed that with the proposed complete immunophenotyping, including the cytoplasmic markers, the frequency of this will be very low and will mostly turn out to be "undifferentiated acute leukaemia" (53). In addition, one should be aware of the bilineage leukaemias, showing two separate cell populations with different distinct features.

In acute leukaemia, it is of extreme importance to search for aberrant markers, since leukaemia-specific combinations offer a powerful tool for minimal residual disease (MRD) detection. In AML TdT, CD19, CD7 and CD56 have been proven to be useful (54, 55, 56, 57, 58). In ALL CD13, CD33 and CD15 are of interest (59). The prognostic importance of MRD detection by immunophenotyping has been proven in several studies (60, 61, 62, 63, 64, 65, 66).

Finally in paediatric acute leukaemias the DNA-ploidy evaluation has prognostic implications (32), and its evaluation is strongly recommended.

4. HAEMATOPOIETIC PROGENITOR COUNTS

An application that has thoroughly changed the logistics of flow cytometric facilities is the enumeration of hematopoietic progenitors in peripheral blood and apheresis samples. The hematopoietic progenitors are being mobilised in cancer patients by either chemotherapy and/or hematopoietic growth factors. Optimally timed peripheral blood stem cell harvesting requires rapid deployment of apheresis and flow cytometry lab staff. The forum participants agreed to recommend the double label procedure (evaluation of CD45 and CD34) described by the International Society for Hematotherapy & Graft Engineering (ISHAGE) (21) because the CD45 measurement provides the necessary quality control for correct RBC lysis.

According to the expertise of some participants, washing is not necessary and thus direct measurement in the lysing solution can speed up the measurements as well as lower the risk for selective losses that may occur during washing. For the calculation of absolute progenitor content the WBC count has to be carefully and immediately carried out on a haematology counter. As an expensive alternative to these double platform measurements a single platform – and thus inherently more precise- flow cytometric measurement can be considered (67): these are based on the simultaneous acquisition of beads with a known concentration, such as TruCount/ProCOUNT (Becton Dickinson) or Flow-Count Fluorospheres (Coulter).

5. AIDS AND OTHER INFECTIOUS DISEASES

The concentration of CD4+ T cells in peripheral blood is considered as one of the most significant prognostic parameter in follow-up of the immune status of HIV-infected persons. Furthermore, this parameter has been shown to be a valuable tool for the evaluation of efficacy of new drugs for the treatment of HIV disease.

Taking into consideration the international recommendations for immunophenotyping of peripheral blood in HIV infection (25), and the Belgian testing limitations (68), we recommend the use of a minimal informative panel of monoclonal antibodies in combination with the whole blood lysing procedure.

For two colour systems, the combinations are CD3/CD4 and CD3/CD8. For three colour systems, the preferred combinations are CD3/CD4/CD45 and CD3/CD8/CD45 rather than CD3/CD4/CD8 alone. For four

colour systems, the combination is CD3/CD4/CD8/CD45. The use of two tubes with a CD3 repeat (two colours) or a CD3/CD45 repeat (three colours) improves quality control. Determining the recovery and purity of the lymphocytes in the gate requires an internal consistency check (see table 1).

To obtain an absolute CD4+ T cell count, the percentage CD4+ T cells (as percentage of lymphocytes) has to be multiplied by the absolute white blood cell count and the percentage lymphocytes obtained from the differential count. Consequently, errors from three different variables are multiplied resulting in a less reliable result. Different solutions to this problem have been proposed, like the use of reference beads (e.g. TruCount approach) or like gating on all white blood cells (CD45) instead of on lymphocytes. The latter, however, requires an extreme fresh blood sample to guarantee granulocyte integrity, cells which are very fragile and die off very quickly after venipuncture. Generally, it can be said that absolute CD4+ T cell count variations are only significant if the percentage of CD4+ T cell changes as well.

6. DISCUSSION

The authors and discussion forum participants hope to have provided the present and future Belgian flow cytometry users and prescribing physicians with a set of valuable consensus guidelines in order to obtain the maximum amount of accurate diagnostic information from the minimal necessary amount of antigenic evaluations.

It is the opinion of all the forum participants that the Belgian refunding system, based on a reimbursement per evaluated antigen of a limited number of antigens (68), regularly creates conflicts and dilemmas for the laboratory professional trying to generate this information. In many respects, the reimbursement is a gross underestimation of the real costs and in the context of a potentially serious disease, these costs are frequently negligible. A reimbursement policy better reflecting the real costs and more adapted to each pathological condition should be more tenable.

During the discussion forums it became clear that there exists hesitation to implement procedures such as triple label staining and cytoplasmic staining which have proven to yield important additional information (not at the least in MRD detection). The routine use of these procedures could be hampered by the lack of expertise and interlaboratory comparison. In order to increase confidence in these procedures the BVC/ABC considers elaborating on this during future workshops and quality control sessions.

SUMMARY

This paper summarises the guidelines and recommendations that were generated during a number of discussion forums attended by the majority of Belgian cytometry laboratory professionals. These forums focused on the rational and optimal use of flow cytometric evaluations in the clinical laboratory setting. The aim was to improve the coherence of the testing panels and the quality of the results and -as such-the clinical diagnostic information. It was also the aim to provide the Belgian prescribing physician and interested laymen with an updated overview of the flow cytometric possibilities. Emphasis is placed on immunophenotyping of haematological malignancies, hematopoietic progenitor cell counting and follow-up of the viral infection caused by the human immunodeficiency virus.

SAMENVATTING

De meerderheid van de Belgische cytometrie laboratoriumverantwoordelijken hebben een aantal discussieforums bijgewoond gecentreerd rond het rationeel en optimaal gebruik van flowcytometrische evaluaties binnen het geheel van de klinische laboratoriumbepalingen. Deze forums hadden tot doel om zowel de coherentie van de testlijsten, als de kwaliteit van de resultaten en – als dusdanig – ook de kwaliteit van de klinisch diagnostische informatie, te verbeteren. Dit artikel beoogt het samenvatten van de gedurende deze forums ontwikkelde richtlijnen en aanbevelingen. Terzelfdertijd hopen we de voorschrijvende artsen en de geïnteresseerde leek een actueel overzicht van de flow cytometrische mogelijkheden ter hand te stellen. De nadruk ligt op het immunofenotyperen van hematologische maligniteiten, op de telling van hematopoiëtische voorlopercellen en op de opvolging van infectie door het humaan immunodeficiëntie virus.

RÉSUMÉ

Différents forums de discussion auxquels ont participé la majorité des laboratoires Belges de cytométrie en flux ont permis de dégager des lignes de conduite et des recommandations décrites dans cet article. Ces réunions avaient pour but de définir les conditions d'utilisation rationnelle et optimale des évaluations par cytométrie en flux dans l'arsenal des tests de laboratoire. Les résultats de ces discussions devraient permettre non seulement d'améliorer la cohérence des panels d'anticorps utilisés, la qualité des résultats et donc l'information diagnostique, mais aussi de fournir aux médecins prescripteurs belges et aux profanes intéressés, une revue des possibilités actuelles de la cytométrie en flux. L'accent a été placé sur l'immunophénotypage des hémopathies malignes, la numération des cellules souches hématopoïétiques, le suivi de l'infection par le virus de l'immunodéficience humaine.

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