Development of Simplified, Inexpensive Flow Cytometry for CD4+ Cell Counting

Bruno Brando, Barbara Scarpati and Giovanna D'Avanzo.
Transplant Immunology and Haematology Laboratory, Falck Building,
Niguarda-Ca'Granda Hospital I-20162 MILANO, Italy.

Counting the absolute numbers and percentage values of CD4+ T Cells in HIV-infected people can be accomplished by different flow cytometric techniques (***Figure 1***). All counting methods listed here have been technically validated, compared with each other and proved to provide similar CD4 counts with only minimal, if any, deviations. It is striking that the same analytical results can be obtained by methods which are remarkably different from each other in their basic design, biological premises and technical details such as the cell identification (gating) methods, monoclonal antibody usage, as well as the numbers of reagents and tubes used.

These diagnostic assays provide a wide range of information on lymphocyte subsets, in line with the complexity of the assays. Still, we must stress that physicians and clinical immunologists who manage infectious diseases are genuinely interested only in the absolute CD4+ cell counts and, particularly in children, in the percent of CD4+ lymphocytes. Clinicians may also find the CD4/CD8 ratios informative during monitoring therapy. Clearly, other parameters including, CD3+, CD19+, CD16 /56+ and CD45 cell levels are not requested and remain largely irrelevant (clinically). An indication of this fact is that these parameters are not included in the clinical protocols for the surveillance of HIV infected individuals.

We, therefore, need to pose the question that **why a simple laboratory analyte like CD4+ T lymphocyte counts require such a complicated array of ancillary tests?** A possible answer is that when the flow cytometry of human lymphocyte subsets was a relatively new investigation many technical problems (such as the inevitable consequences of separating cells with Ficoll-Hypaque, using indirect immunofluorescence methods and applying cycles of washing cells, etc.) contributed to the great variations observed. For these reasons, abundant measures have been introduced to check internal consistency and to avoid major errors, e.g. by confirming the credibility of CD4 results by inspecting other cell lineage markers as well. Nevertheless, the technology of flow cytometry has brilliantly improved since those early days. In addition, with the arrival of the HIV mediated catastrophe in the developing world **there is a powerful clinical incentive to have a fresh look**, as Sherman and Glencross have done in their important paper (see selected references).

One then ask whether or not recent improvements in flow cytometry could lead to a simpler technology. It is a common practice in clinical chemistry, serology and haematology that analytes are determined as **a single variate requiring only the appropriate calibrators and standards. Can the same concept be now applied to CD4+ cell count?** The main criterion in CD4+ cell enumeration is the discrimination between the high density of CD4 staining on a lymphocyte subset and the

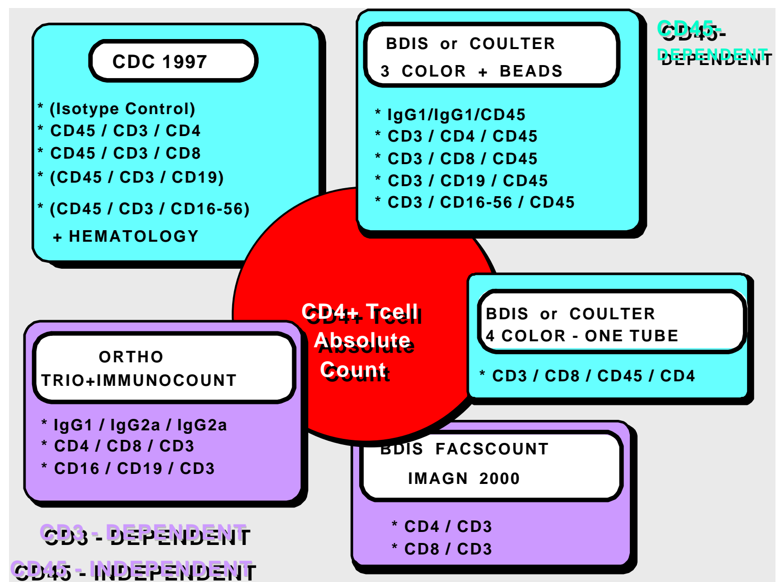

Figure.1 *The various parameters investigated, routinely and according to the recommended protocols, during the current assays for CD4 absolute counts. The accuracy, reproducibility and correlations of these techniques have been established (reviewed by Brando et al. Cytometry 2000).*

lower density of CD4 staining on monocytes. Modern flow cytometers with high resolution fluorescence detection and right-angle scatter (SSC) are able to identify and enumerate CD4+ cells with a single-colour staining technique and therefore greatly reduce the complexity of the assay.

We undertook a series of experiments to validate this concept. The term *"Full Technology"* refers to multiple-colour, single- or multiple tube immunophenotyping for lymphocyte subsets according to recommended protocols (reviewed by Brando et al. *Cytometry* 2000;). The numbers of reagents included in this technology is shown in Figure 1. The term *"Minimal Technology"* refers to techniques that utilise simplified methods for staining and/or analysis in order to reduce assay complexity and inherent costs.

Dr. Bruno Brando's Phone: +39 02 6444 2329 Fax: +39 02 6444-2957 or -2910 e-mail: bruno.brando@iol.it

Comparison Between CD4 Assays performed using *Full-* and *Minimal Technology*

In our study blood samples from 125 HIV+ adult subjects were analysed on single-platform. Absolute CD4 mean values ranged 11-3600 cells/µL. Three- and four-colour Full Technology counting procedures were simultaneously used on Ortho-Cytoron, BD FACSCalibur, Coulter EPICS XL and Dako Galaxy. On the Dako Galaxy volumetric counting was performed using two different staining protocols: *(i)* the same Trio staining as performed on the Ortho Cytoron and *(ii)* additional staining in two tubes with CD3/CD4/CD45 and CD3/CD8/CD45.

From each analysis file the CD4 single-colour dataset was isolated and reanalysed according to the concept of Minimal Technology. Each Absolute CD4 count obtained by Full Technology methods was compared, first, with its own Minimal Technology, and then cross-compared with all Minimal Technology data obtained on the other systems. The Bland-Altman statistics was used throughout.

When each instrument data were compared internally as Full *vs.* Minimal Technology counts, the mean difference (bias) ranged from –2 to +13 with a very narrow standard deviation of the mean difference. These results indicate an excellent agreement between the two approaches as shown in ***Table 1***. These excellent agreements between the two different approaches in the various flow systems indicate that the additional markers and internal checks for consistency do not introduce any particular extra advantage during CD4 analysis performed with these modern flow cytometric systems.

The agreement between the two methods shown in Table 1 may be criticised because data are partly self-referential. Nevertheless, this criticism is no longer valid when each *Full Technology* approach is cross-compared with all the other *Minimal Technology* assays *(**Table 2**)*. Obviously, however, during this comparisons the extra complications of using flow methods based on different principles (see Footnote to Table 2) increase the inherent variations - although by a surprisingly low degree. Here the values of mean differences fall into two groups. In the first six rows the bias was <16 CD4 cells indicating that ORTHO Cytoron, FACS Calibur and Coulter XL gave similar mean values. In the last three rows all results show that DAKO Galaxy under-estimated CD4 counts my a mean of 34 to 47. When the values of the limits of agreement are inspected, these are widened as expected when compared to Table 1. The best agreement was seen between TRUCount and FlowCount methods, and between ORTHO and TRUCount. FlowCount versus ORTHO comparison ranked intermediately. Again, the DAKO Galaxy showed the lowest agreement with all the other analysis systems due to a clear and reproducible bias to underestimate cell counts on the GALAXY *(**Table 2**)*. Similar underestimation of absolute counts is observed on Partec PAS, a flow cytometer of similar design to GALAXY (G. Janossy, personal communication).

The most important issue is that these observations prove that **absolute CD4+ cell counting can be effectively performed with simplified single-colour techniques**. It appears that in adult patients the use of other cell lineage markers (CD3, CD19, CD16) does not represent an appreciable advantage over the minimalist single-colour CD4 approach. Nevertheless, extra staining with CD45 for precise leukocyte and lymphocyte counting helps generate more precise CD4% values, particularly when requested in children. Similarly, CD8 counts are useful for documenting CD4/CD8 ratios. In conclusion, when used in non-oncohaematologic setting, minimal technology is sufficiently precise and accurate to provide clinically relevant absolute CD4+ cell counts.

Table 1

CD4 counts: Full Technology compared with their respective Minimal Technology data

Absolute CD4+ Counting Method	Mean Diff	2 x SD	Limits of Agreement LOWER	Limits of Agreement UPPER	MEAN Range
ORTHO Cytoron ABS using Trio staining	-2	11.1	-13	9	21 - 3600
FACSCalibur using TRUCount	-1	12.5	-13.5	11.5	22 - 3243
Coulter XL using FlowCount	-2	11.1	-13	9	25 - 2796
DAKO Galaxy using Trio staining	13	18.4	-15.4	31.4	11 - 1676
DAKO Galaxy using CD45 staining	-4	7.4	- 11.4	3.4	44 - 1492

Mean Diff = Mean difference of the paired data; **2xSD** = Standard Deviation of the mean difference multiplied by two; **Lower** and **Upper** Limits of agreement are calculated as Mean Diff ± 2xSD, respectively; **Mean Range** = minimum and maximum CD4+ Cell absolute count, as calculated by averaging the paired data. DAKO Galaxy data refer to 88 cases out of 125.

Dr. Bruno Brando's Phone: +39 02 6444 2329 Fax: +39 02 6444-2957 or -2910 e-mail: bruno.brando@iol.it

Table 2 Absolute CD4+ cell counting methods using Full Technology compared with all the other Minimal Technology counting systems

Absolute CD4+ Counting Method	Mean Diff	2 x SD	Limits of Agreement LOWER	Limits of Agreement UPPER	MEAN Range
FULL Tech *vs MINIMAL*					
ORTHO *vs TRUCount*	9	65	-56	74	20 - 3394
ORTHO *vs FlowCount*	-5	84.5	-89.5	79.5	22 - 3383
TRUCount *vs FlowCount*	-12	52	-64	40	24 - 3231
TRUCount *vs ORTHO*	-12	63.5	-75.5	51.5	23 - 3449
FlowCount *vs ORTHO*	7	141.5	-134.5	148.5	24 - 3014
FlowCount *vs TRUCount*	16	45.5	-29.5	61.5	23 - 2808
GALAXY *vs ORTHO*	-47	140	-187	93	18 - 3240
GALAXY *vs TRUCount*	-34	115	-149	81	17 - 3043
GALAXY *vs FlowCount*	-45	116	-161	71	19 - 3031

The methods using Full Technology are compared to CD4 results obtained with the other, different, *Minimal Technologies* (shown here in *italics)*. In these assays, similar bead-based principles are used with the TRUCount and FlowCount. ORTHO and GALAXY both represent volumetric measurements but based on different principles (reviewed by Brando et al. *Cytometry* 2000: in press). The lowest SDs are recorded between TRUCount and FlowCount, in either comparison. Markedly imbalanced mean difference and high SD is evident when DAKO Galaxy is involved, due to a bias of underestimation (between -34 and -47).

The Issue of Clinically Significant Error in Absolute CD4 Counting

In monitoring the CD4 absolute counts we must now take into account a hitherto poorly defined issue, namely the magnitude of the measurement variability that can influence clinical decisions. A preliminary attempt to define the critical error boundaries for absolute T CD4+ cell count has been performed at the S. Martino Hospital/University of Genova, Italy, by Dr. Annalisa Kunkl (akunkl@smartino.ge.it). The data came from comparative analyses of 24 HIV+ and normal blood samples by 18 laboratories participating to a regional QC program. Each sample was analysed on double platform equipments (and therefore the procedure may need to be repeated on single platform systems if the clinicians finds the reported variations uncomfortable high). True CD4+ cell values were defined by the consensus mean; the 99.9% confidence interval of the mean was calculated on the basis of a total of some 300 valid data after the elimination of outliers and data without internal consistency. Regression of the upper and lower confidence limits of the 24 samples were used to extrapolate confidence ranges for any theoretical CD4+ value (Upper Limit regression Line: Y=1.126 AbsCD4 +7.64; Lower Limit Regression Line: Y=0.874 AbsCD4 -7.64).

Confidence intervals of true CD4+ values have been taken as a measure of the error due to the variation of CD4+ counts. As an example, with this method, the 99.9% confidence intervals in the case of measured CD4+ counts of 100 and 300 CD4+ T cells/µL were (80/µL, 120/µL) and (254/µL, 346/µL) respectively. From Kunkl's studies it is also apparent that an observed value of 200 CD4+ T Cells/µL can mean any real CD4 count from 167 to 216/µL, representing 24% variation under optimal analytical conditions. These issues must also be viewed in the light of physiological, diurnal and circannual variability of CD4+ T cell counts .

The estimation of test variability is a prerequisite of any clinical study including threshold values as decisional boundaries. Strangely enough, in almost any instance fixed threshold values, including the 200 CD4+ T cells/µL for an AIDS related boundary value, have been first established by some powerful international committee without pointing out the levels, and the importance, of laboratory variations. The world-wide accepted CDC guidelines on AIDS-defining conditions state that below 200 CD4+ T Cells/µL a HIV-infected subject is classified as having AIDS by laboratory criteria. But the variations of this threshold has not been at that time or ever since officially assessed despite the compelling results shown above following the use of double platform equipments. At this point the assay variability of the CD4 tests performed on single platforms around this threshold value is still unknown.

Consequently, when the analytical variability can still be so wide one must doubt the wisdom of investing time, money and technology into the propagation of apparently over-complicated assay procedures for CD4+ cell counting. Furthermore, this analytical variability is just one of the many components that contributers to the total assay variability. The additive factors include 'center effect',

Dr. Bruno Brando's Phone: +39 02 6444 2329 Fax: +39 02 6444-2957 or -2910 e-mail: bruno.brando@iol.it

patient status, drug treatment, concomitant disease and diurnal as well as circannual variations.

In conclusion, for the full clinical use of CD4 tests four factors are important:

(i) Affordable, simple CD4 assays,

(ii) an understanding of clinical and biological factors during the interpretation of results,

(iii) to appreciate assay variability at the boundaries of CD4 values that lead to clinically important decisions, and

(iv) quality assurance programs for decreasing laboratory variations and securing training as well as the uniformity of data handling.

Selected References:

Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'Hautcourt JL, Lenkei R, Schmitz G, Kunkl A, Chianese R, Papa S, Gratama JW. Cytofluorometric methods for assessing absolute numbers of cell subsets in blood. *Cytometry (Communications in Clinical Cytometry),* 2000; 42: 327-346.

Brando B, Sommaruga E, Mazzola E, Sbressa C. CD4+ T Cell absolute count. A crossed comparison among three flow cytometric methods and plate ELISA test in 325 HIV+ patients. *Cytometry* 1996; Suppl. 8: Abstract IH32, pg 77.

Brando B. Development of a 'minimal technology' procedure for absolute counting of CD4+ cells in HIV+ patients. *Cytometry* 2000; Suppl. 10: 138 (Abstract 6390).

Janossy G, Jani I, Göhde W. Affordable CD4+ T cell counts on 'single-platform' flow cytometers. I. Primary CD4 gating. *Br J Haematol*, 2000; 111: 1198-1208.

Sherman GG, Galpin JS, Patel JM, Mendelow BV, Glencross DK. CD4+ T cell enumeration in HIV infection with limited resources. *J Immunol Methods* 1999; 222: 209-217.

Kunkl A, Terranova MP, Mortara L, Bottone. Measurement error of absolute CD4+ T lymphocyte count double platform evaluations for the assessment of laboratory performance in lymphocyte immunophenotyping quality control. *Cytometry* Suppl. 10, 2000: 45, Abstract 6415.

Hoover DR, Graham NMH, Chen B, Taylor JMG, Phair J, Zhou SYJ, Munoz A. Effect of CD4+ cell count measurement variability on staging HIV-1 infection. *J Acquir Immune Defic Syndr Hum Retrovirol* 1992; 5: 794-802.

Paglieroni TG, Holland PV. Circannual variation in lymphocyte subsets, revisited. T*ransfusion* 1994; 34: 512-516.

Hughes MD, Stein DS, Gundacker HM, Valentine FT, Phair JP, Volberding PA. Within-subject variation in CD4 lymphocyte count in asymptomatic human immunodeficiency virus infection: implications for patient monitoring. *J Infect Dis* 1994; 169: 28-36.

Raboud JM, Haley L, Montaner JS, Murphy C, Januszewska M, Schechter MT. Quantification of the variation due to laboratory and physiologic sources in CD4 lymphocyte counts of clinically stable HIV-infected individuals. *J Acquir Immune Defic Syndr Hum Retrovirol* 1995; 10 Suppl 2: S67-S73.

Jones AR, Twedt D, Swaim W, Gottfried E. Diurnal change of blood count analytes in normal subjects. *Am J Clin Pathol* 1996; 106: 723-727.

Dr. Bruno Brando's Phone: +39 02 6444 2329 Fax: +39 02 6444-2957 or -2910 e-mail: bruno.brando@iol.it