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Biological variability of lymphocyte subsets of human adults' blood

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ABSTRACT

Background: Alterations in lymphocyte subpopulations are present in several immune diseases, and clinicians and researchers recognise the importance of investigating the distribution and changes in lymphocyte subsets over relatively long periods of time in order to evaluate the effectiveness of treatment and follow the course of disease. Yet further insight is required on the biological variability (BV) of lymphocyte subsets, which is crucial to the correct interpretation of longitudinal changes and provides essential information for setting desirable quality specifications and defining the usefulness of reference values.

Methods: Four-colour-flow cytometry was used to investigate the BV of lymphocyte populations (LP) in the peripheral blood of 20 healthy adults recruited from our laboratory staff and followed for three months. The total lymphocyte count was measured, and the relative frequencies determined for T-cells (CD3+), T-helper cells (CD3 + CD4+), cytolytic T-cells (CD3 + CD8+), B-cells (CD3 − CD19+), NK-cells (CD3 − CD16+/56+), non-MHC restricted cytolytic T-cells (CD3 + CD56+) and activated T-cells (CD3 + HLA-DR+).

Results and conclusions: Data on the components of BV were applied to set quality specifications for allowable precision, bias and total error. Analytical performances were established, and they were more than desirable for all the markers considered in our study.

By comparing within-subject and between-subjects BV, we were able to define the uselessness of reference ranges in the evaluation of changes in CD serial results. Data on within-subject BV and analytical precision were thus used to determine the reference change values, in order to identify the significance of changes in serial results.

The findings made in the present study provide further evidence of the relevance of BV in the evaluation of immunological markers of LP.

1. Introduction

Lymphocytes have both regulatory and effector roles and the distribution of the different populations in peripheral blood can be altered in many primary immune diseases or diseases with secondary involvement of the immune system. To follow the course of disease and evaluate the effectiveness of treatment, the distribution of, and changes in, the lymphocyte populations (LP) are often monitored for long periods, both for clinical and research purposes.

In order to accurately estimate longitudinal changes it is important to take into consideration biological variability (BV) of the measurement (i.e. random fluctuation around a homeostatic setting point, the so-called within-subject or intra-individual BV). Pure estimates of the average within-subject BV and the between-subjects BV are obtained by carefully controlling pre-analytical variability and by designing experiments that allow the quantification of analytical variability [1].

Pre-analytical variability should be minimised as far as possible. Therefore candidates for study must be: willing to provide a number of samples over a long period of time, healthy, and not on any drugs that might affect the analytes under investigation; nor should they have unusual lifestyles or habits, or have an alcohol intake in excess of the units specified [1].

BV can be used to: set quality specifications; consider the utility of conventional population-based reference values; determine the change occurring in an individual's serial results before it becomes significant (reference change values (RCV)) [1].

A number of early studies available in literature evaluating LP BV reported variations occurring during a day [2–6] and/or during a year [7,8,3,9–11]. More recent studies demonstrate that the circadian variability of lymphocyte subsets is very relevant, and correlates with the neuro-endocrine system and changes with aging [12–14].

However, in spite of its well-defined clinical role, no reliable data on the BV occurring in lymphocyte subsets have yet appeared in literature.The present study therefore reports findings obtained by making a definitive assessment of the analytical and biological components of variability of lymphocyte subsets using an accurately designed experimental and statistical protocol.

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2. Materials and methods

2.1. Subjects

A valid estimate of the components of variability is obtained from relatively small numbers of specimens collected from a small group of subjects over a relatively short period of time [1]. Therefore, over a three-month time-period (February 2012–May 2012), four blood specimens were drawn from each of 20 healthy volunteers (11 women, 9 men; age range, 25–58 years) on the same day, at regular time intervals (start time, after 1 week, 1 month and 3 months).

Apparently healthy subjects were studied to ensure that any LP fluctuation in peripheral whole blood would truly reflect biology and not modifications due to pathological processes. In accordance with the Helsinki II Declaration, the design and execution of the experiment were explained thoroughly to the subjects, and fully informed consent was obtained in writing.

2.2. Experimental design

The group of subjects selected for studies evaluating BV should be considered "reference individuals"; in order to minimise pre-analytical variability, exclusion or inclusion criteria are established before the accrual of subjects [1].

Since its aim was to obtain data on BV, our experimental protocol minimised the numerous pre-analytical factors that can influence the results of the tests, such as lifestyle, time of sampling, phlebotomist, specimen handling and storage [1].

The subjects meeting inclusion criteria had no current illness or infection, were non-smokers, had not exercised, and had neither taken medication nor consumed substantial quantities of alcohol; none of the women were pregnant. During the study period, subjects were asked to continue with their dietary habits and usual activities. To minimise circadian variability in subjects, venous blood was obtained within the same time-period of the day (between 09.00 and 10.00). The same phlebotomist collected blood samples with minimal stasis into vacuum collection tubes with EDTA as the anticoagulant (Becton-Dickinson, Milan, Italy). The total lymphocyte count of all the subjects was within the reference range (1100–4800/μL). Samples were stored at room temperature until staining.

2.3. Methods

The total lymphocyte count was measured with an ADVIA 2120 haematology analyser (Siemens).

The following 4-colour combinations of monoclonal antibodies (Beckman-Coulter) were used: CD45-FITC/CD4-PE/CD8-ECD/CD3-PeCy5, CD45-FITC/CD56-PE/CD19-ECD/CD3-PeCy5, and CD16-FITC/HLA-DR-PE/CD3-ECD/CD45-PeCy5.

Sample preparation was performed according to the NCCLS (H42-A2) guidelines [15]. In particular, 100 μL of EDTA blood was added at analiquot of monoclonal antibody as suggested by the manufacturer, and incubated for 15 min at room temperature. After incubation, erythrocytes were lysed with TQ-prep (Beckman-Coulter). Samples were then acquired on the flow cytometer.

Staining was always undertaken on the day of sampling, within 3 h. Four-colour-flow cytometric analysis was performed on a Navios (Beckman-Coulter). Acquisition was run until 25,000 events were detected. Data analyses were made with CXP software.

Using the appropriate "gating" approach, the relative frequencies were determined for T-cells (CD3+), T-helper cells (CD3 + CD4+), cytolytic T-cells (CD3 + CD8+), B-cells (CD3 − CD19+), NK-cells (CD3 − CD16+/56+), non-MHC restricted cytolytic T-cells (CD3 + CD56+) and activated T-cells (CD3 + HLA-DR+). Since we work with a dual platform, the absolute frequencies of the cells subsets were calculated on the basis of the relative frequencies on total lymphocytes.

Internal quality control (IQC), the so-called Immunotrol, performed daily, consisted of a liquid preparation of human stabilised red and white blood cells that was processed as a routine sample with the reagents in use. Before being included in routine use, the new batch of Immunotrol was analysed simultaneously with the old batch for at least three days, to determine whether any outside range results should have been attributed to unsuitability of the new batch or to other causes.

The control values were stored in the instruments in a specific file of the program called Quality Control (QC).

If the IQC was within the acceptable range, sample acquisition and analysis were performed.

The instrument was calibrated, according to the operational manual, once a month and also whenever there were problems with the IQC or instrumental breakdowns occurred. If the calibration was not accepted, it was repeated using a new calibrator and, if it was still not acceptable, external technical assistance was sought.

The calibrator was processed as indicated on the package insert, and the calibration was stored in the instruments. All the calibrators were conserved in cold room at radio controlled temperature.

The UK Neqas External Quality Assessment "Leucocyte Immunophenotyping" external QC was used for assessing CD3, CD4, CD8 and CD19 values.

3. Statistical analysis

All data were investigated for outliers. Cochran's Q test was performed for outlier identification among observations and within-subject variations, whereas Reed's criterion was used to identify outliers among the mean values of subjects [1]. After outlier exclusion, the Kolmogorov-Smirnov test was applied separately to the set of results from each individual to check data distribution.

The analytical, within-subject, and between-subjects components of variability were calculated by nested ANOVA from replicate analyses [1].

Within-subject BV was estimated from the within-subject total variability minus one-half of the analytical variability, and between-subjects BV from the total variability of the data minus the analytical and intra-individual components.

Precision was calculated by means of the IQC program of the laboratory.

In order to assess the analytical uncertainty of laboratory results and provide a more correct clinical interpretation and utilisation of results, the analytical total error was calculated for each analyte as follows: bias + 1.65 CVa, where bias was the mean of bias (result − target value/target value*100) obtained at significant clinical concentrations, in an annual cycle, from reports of the External Quality Assessment Scheme; CVa represents the analytical variation coefficient (CVa%) obtained by determining internal control materials at clinically significant concentrations [1].

The index of individuality (II), a ratio calculated as $(SDintra^2/SDbetween^2)^{1/2}$, allowed the objective assessment of the value of population-based reference values. When the II is greater than 1.4, the conventional population-based reference values are relevant. When the ratio is low, particularly less than 0.6, conventional population-based reference values are of little relevance in the correct evaluation of results because individuals may have marked changes in marker levels that are significantly different from their own usual values and all results may still lie within the usual reference interval [16].

The index of heterogeneity (IH) was calculated as the ratio between the observed CV of the set of individuals (including analytical) to the theoretical CV, which is $(2/k - 1)^{1/2}$, k being the number of specimens collected per subject. If the index differed from 1.0 by more than 2