ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA
VOL. LV, 13 SECTIO D 2000

Department of Clinical Immunology, University School of Medicine, Lublin
Zakład Immunologii Klinicznej Akademii Medycznej w Lublinie

JACEK TABARKIEWICZ, MICHAŁ DRAGAN,
PAWEŁ KRAWCZYK, JACEK ROLIŃSKI

Identification and enumeration of myeloid dendritic cells from peripheral blood. Comparison of two protocols: in whole blood and in isolated mononuclear cells

Oznaczanie jakościowe i ilościowe mieloidalnych komórek dendrytcznych krwi obwodowej. Porównanie dwóch procedur oceny: w krwi pełnej i w izolowanych komórkach mononuklearnych

INTRODUCTION

Dendritic cells (DC) are potent antigen presenting cells (APC) that are essential for the initiation of primary immune response. They are believed to play a crucial role in the initiation and maintenance of T–cell immunity [1, 2, 3]. DC are present in lymphoid and non lymphoid tissues [1]. Many studies were carried out to understanding the role these cells in the physiologic immune response and in the pathogenesis of human diseases. DC are obviously linked with infectious diseases and malignancy. There is a steadily increasing body of literature correlating DC numbers with skin carcinogenesis, cervical carcinoma, colorectal adenocarcinoma, gastric carcinoma, nasopharyngeal carcinoma, lung adenocarcinoma, thyroid and prostate cancer [1]. Increased numbers of dendritic cells are associated with better outcomes in mentioned malignancy diseases [1]. There have been reports of connection between DC and viral infections e.g. HIV, influenza, herpes simplex [1]. Also bacterial, protozoal and fungal infections are connected with antigens presentation by dendritic cells [1]. DC play a role in autoimmune diseases, immunodeficiency, hypersensitivity [1]. These findings suggest, that dendritic cells could be used in the treatment of human diseases. Current investigations are focused on using DC for immunotherapy of malignant or infectious diseases. The ability to identify and enumerate DC is fundamental to understanding the role, which these cells may play in the pathogenesis and treatment of human diseases. Peripheral blood dendritic cells are an important and readily accessible cell populations to repeated sampling. Immunostaining directed against mul-

tiple cell surface antigens and flow cytometry methods are used for identification of circulating DC. The problem hampering the identification of circulating DC is the absence of a single DC–specific cell marker. Freshly isolated blood DC do not usually express CD1a or CD1c [3, 4, 6] and they are negative for the DC–specific antigens CD83 6 and CMRF–44 [5]. Previous investigations have shown, that myeloid DC could be identified as CD33+, HLA–DR+, CD14–, CD16– cells [3, 4] and this method was used in our research. We investigated if manipulation of blood samples would affect the number of collected myeloid DC.

MATERIAL AND METHODS

Peripheral blood was obtained from 13 healthy donors. Informed consent was obtained from each individual. The cell surface antigens in each case were determined on fresh cells at the time of sample submission. The following directly conjugated monoclonal antibodies (mAbs) were used: mouse anti-human CD14–FITC (Dako, Denmark), CD16–FITC (Dako, Denmark), CD33–PE (Dako, Denmark) HLA–DR–CyChrome (Pharmingen, USA). Examined blood was divided into two parts: for whole blood procedure and for isolation of mononuclear cells.

One hundred microliters of whole blood was added to 5–ml polystyrene tube (Falcon) and incubated with mAbs at 4°C for 30 min. Red cells were lysed by addition of FACSlyse Solution (Becton Dickinson) according to BD's manual. After lysing, cells were washed twice in phosphate buffered saline (PBS) and analysed using a flow cytometer. We collected 1,000 DC or 300,000 of total events.

Mononuclear cells from second part of blood were isolated on gradient density centrifugation (Lymphoprep, Nycomed–Norway). Isolated cells (10^6 of cells per tube) were incubated with mAbs at 4°C for 30 min and washed before flow cytometry analyse.

For both protocols cells were collected using a FACSCalibur flow cytometer equipped with 488–nm argon laser (Becton Dickinson) and analysed with CellQuest Software.

Wilcoxon-non-parametric test and Statistica 5.0 PL software were applied to statistical analysis.

RESULTS

Previous researches have shown, that CD14–, CD16–, CD33+, HLA–DR+ cells from peripheral blood possess features typical for myeloid DC [3, 4]. In our study, we identified the same population of cells by immunofluorescence staining and flow cytometry analysis of small quantities of lysed whole blood (Fig. 1) and isolated cells (Fig. 2). At first, we gated out CD14 and CD16 positive cells, then we identified a small cell population that strongly express both HLA–DR and CD33. This population was previously described as myeloid dendritic cells. J.W. Upham et al. [3] found, that it is necessary to use CD14 and CD16 simultaneously in the staining protocol, in

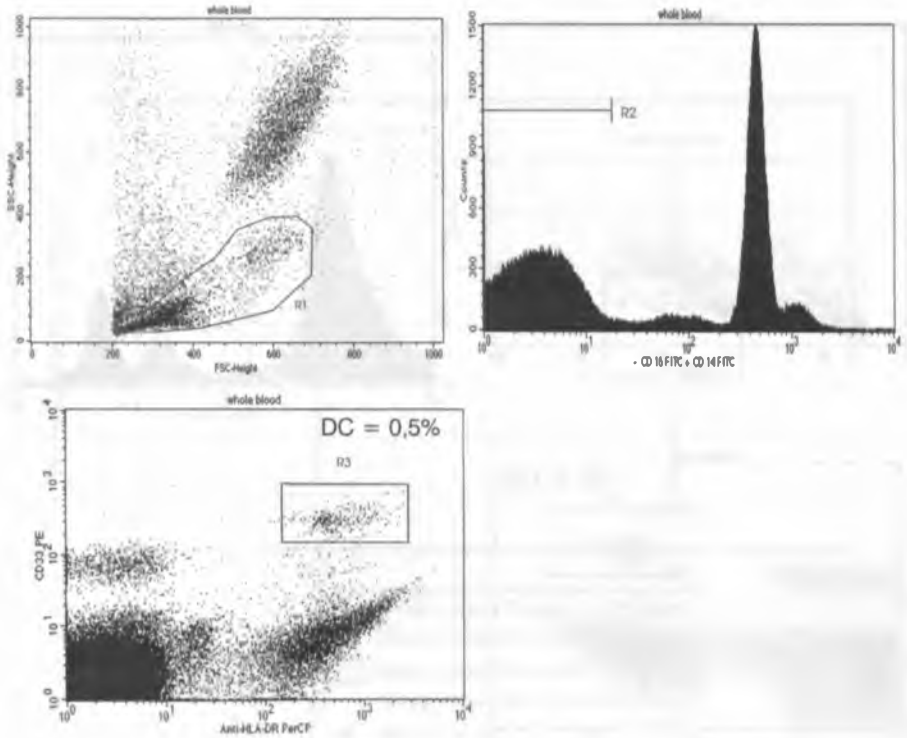

Fig. 1. Identification of circulating CD33+DC by flow cytometry using whole blood protocol. A: The mononuclear cells analysis region (R1) applied to FSCxSSC. B: The R1 gated events were then analysed for CD14 and CD16 staining and negative cells were gated (R2). C: Myeloid DC were identified as cells expressing high levels of HLA–DR and CD33, but without expression of CD14 and CD16 (R3)

order to exclude from the analysis typical monocytes (CD14high and CD16–) and the monocytes that coexpress CD14 and CD16. Because of low frequency of circulating myeloid DC, it was important to acquire a large number of events within the mononuclear cell light scatter region in order to provide more accurate DC numbers. The count of DC were expressed as a proportion of DC to peripheral blood mononuclear cells (PBMC).The average number of DC was 0.32% ±0.11 of PBMC for whole blood protocol and 0.32% ±0.13 of PBMC for isolated cells. Using the Wilcoxon-non-parametric test, we found that was no statistically significant difference ($p = 0.753$) between number of CD33+/HLA–DR+ cells stained and counted by whole blood protocol and protocol with isolation on density gradient centrifugation (Fig. 3).

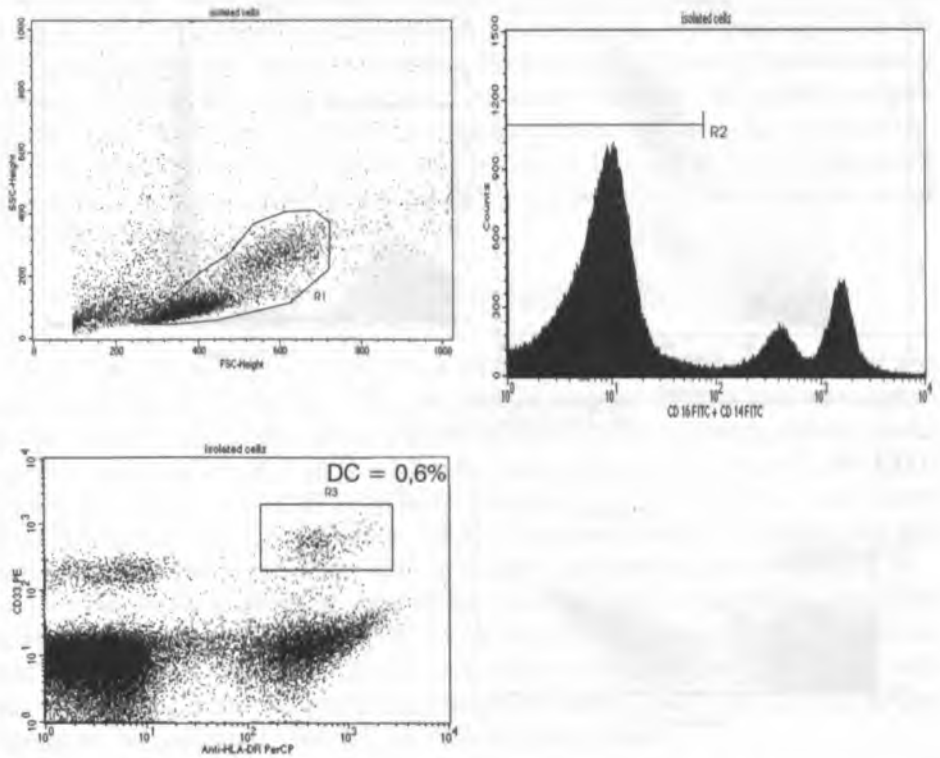

Fig. 2. Identification of circulating CD33+DC by flow cytometry using isolation on gradient centrifugation. A: The mononuclear cells analysis region (R1) applied to FSCxSSC. B: The R1 gated events were then analysed for CD14 and CD16 staining and negative cells were gated (R3). C: Myeloid DC were identified as cells expressing high levels of HLA–DR and CD33, but without expression of CD14 and CD16 (R3)

DISCUSSION

Method used in our study is simple three-colour protocol for the identification of the major subset of circulating myeloid DC using commercially available reagents. Previous investigations have shown that CD33+, CD14–, CD16–, HLA–DR+ cells purified from peripheral blood possess many features of DC [3, 4]. Following a short period of in vitro culture, they develop typical dendrites and increase expression of various costimulatory molecules [3]. CD33+ DC have light scatter characteristic similar to myeloid DC previously shown expression of CD11c [3], which is one of the antigens used for identification of DC. J.W. Upham et al. [3] have compared this method with commercial kit developed by Becton Dickinson (BD) for identification of DC subset in peripheral blood. In BD assay, DC are defined as HLA–DR+, lineage-negative cells (lacking CD3, CD14, CD16, CD19, CD20, CD56) and which express either CD11c or CD123. There was no significant difference between the fre-

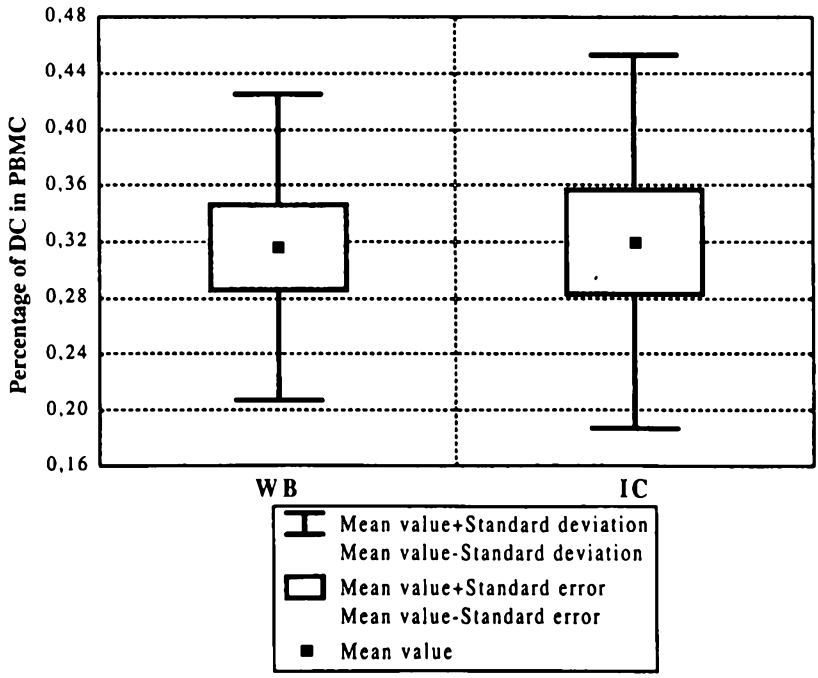

Fig. 3. Percentage of DC. Comparison between whole blood (WB) protocol and isolated cells (IC)

quencies of CD33+DC obtained by method used in their study and CD11c+, lineage-negative DC obtained using commercial kit. The other scientists have also estimated that DC number comprise between 0.1% and 2.0% of PBMC [3, 4, 5, 6], although they used different mAbs for identification of DC. Some investigators employed extensive cell separation and enrichment procedures. In our study we have shown, that there was no statistically significant difference ($p = 0.753$) between whole blood protocol and protocol including cells isolation. Additionally, we concluded that standard deviation was smaller, when CD33+DC were obtained from whole blood without cell isolation. Method used in our study is reproducible, rapid, less costly and requires small volumes of blood. Our results revealed, that cell isolation does not influence the number of marked and counted CD33+DC. On the other hand, isolated cells are more suitable for detailed identification of surface antigens, e.g. markers of activation (CD69, CD83, CD25), costimulatory molecules (CD40, CD80, CD86), that allows us more detailed analyse of DC subsets. Researches carried out around the world are focused on methods allow enumeration of absolute DC counts in peripheral blood, establishment of normal ranges of circulating DC. Detailed examina-

tion of the relationships between various pathological conditions and numbers of circulating DC are necessary for better understanding of the role that dendritic cells play in pathogenesis of human diseases. Clinical studies based on DC immunotherapy started few years ago [2].

CONCLUSIONS

We concluded that:

1. Identification of myeloid DC as CD33+, CD14–, CD16–, HLA-DR+ cells is reproducible, rapid and less costly method.
2. Enumeration of DC by this method gives results close to other methods.
3. Isolation of cells on Ficol gradient does not interfere in the number of detected CD33+DC.
4. Standard deviation was smaller when cells were stained and counted in lysed blood.

REFERENCES

1. *Hart D.N.J.*: Dendritic cells: unique leukocyte populations which control the primary immune response. Blood, 1997; 90: 3245–3287.
2. *Avigan D.*: Dendritic cells: development, function and potential use for cancer therapy. Blood Reviews, 1999; 30: 51–64
3. *Upham J.W., Lundahl J., Liang H. et al.*: Simplified quantitation of myeloid dendritic cells in peripheral blood using flow cytometry. Cytometry, 2000; 40: 50–59.
4. *Thomas R., Lipsky P.E.*: Human peripheral blood dendritic cell subsets. Isolation and characterisation of precursor and mature antigen presenting cells. J. Immunology, 1994; 153: 4016–4028.
5. *Fearnley D.B., McLellan A.D., Mannering S.I., Hock B.D., Hart D.N.J.*: Isolation of human blood dendritic cells using the CMRF–44 monoclonal antibody: implications for studies an antigen presenting cell function and immunotherapy. Blood, 1997; 89: 3708-3716
6. *Zhou L.J., Tedder T.F.*: Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J. Immunology, 1995; 154: 3821–3853.

STRESZCZENIE

Komórki dendrytyczne (DC) są komórkami prezentującymi antygen (APC), które inicjują pierwotną odpowiedź immunologiczną zależną od limfocytów T. Poprzednie badania donoszą, że DC biorą udział w patogenezie wielu chorób człowieka. Krew obwodowa pobrana była od 13 zdrowych dawców. Krew pełna jak i izolowane komórki inkubowane były z przeciwciałami monoklonalnymi anty–CD14, anty–CD16, anty–CD33, anty–HLA–DR. Określiliśmy odsetek DC w komórkach mononuklearnych krwi obwodowej. Odsetek ten dla protokołu z użyciem krwi pełnej wynosił średnio 0, 32% ±0,11, a dla komórek izolowanych 0,32% ±0,13. Nie stwierdzono statystycznie istotnej różnicy w odsetku komórek dendrytycznych znakowanych z krwi pełnej i z komórek izolowanych. Izolacja komórek mononuklearnych nie wpłynęła na uzyskany odsetek DC. Identyfikacja mieloidalnych komórek dendrytycznych jako CD33+, CD14–, CD16–, HLA–DR+ jest powtarzalną, szybką i mało kosztowną metodą, która może być pomocna w ocenie ich potencjału terapeutycznego.