Immunobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Research paper

Accelerated reactivity of blood granulocytes in patients with atopic bronchial asthma out of exacerbation

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ARTICLE INFO

Key-words:
Phagocyte
Respiratory burst
Kinetics
Receiver operating characteristic
Priming
Adhesion
Allergic bronchial asthma

ABSTRACT

Reactive oxygen species (ROS) are important in bronchial asthma (BA) pathogenesis owing to accumulation of activated granulocytes in the lungs. But the ROS-producing activity of the cells is insufficiently understood in the blood of BA patients. This study analyzes the kinetics of phagocyte respiratory burst in the blood to improve the methods of BA patients monitoring. Patients with atopic BA out of exacerbation (n = 60) and healthy controls (n = 43) were recruited. The time-course of respiratory response to opsonized zymosan (OZ) was recorded in the whole blood using luminol-dependent chemiluminescence (CL), and its activation kinetics (lag-time, rate, amplitude, ROS production) was calculated. The discriminative power of ROS generation kinetics was defined by Receiver Operating Characteristic (ROC) curve analysis. Standard physiological respiratory parameters of patients did not differ from the controls. More rapid response to OZ was recorded in BA patient samples versus the controls. The primed state of phagocytes in the blood of BA patients was corroborated by significant weakening formyl peptide priming effect. The adhesion of granulocytes to cultured human endothelial cells was two-fold higher in BA patients versus controls. ROC curve analysis exhibited good discriminative effectiveness of the CL kinetics to compare BA individuals with the controls. The highest power (86% sensitivity and 90% specificity) was achieved at a linear combination of the parameters. We assume that the assessment of phagocyte reactivity based on the analysis of the response kinetic profile is a good test for monitoring of the state in BA patients.

1. Introduction

Bronchial asthma (BA) is a heterogeneous disease, usually characterized by chronic airway inflammation (Global Initiative for Asthma, 2015; Hinks et al., 2016; Koczulla et al., 2017). The heterogeneity of asthma with different phenotypes and endotypes is one of the obstacles to successful management (Agache and Akdis, 2016). The clinical phenotype of atopic BA is the most recognizable one, since it is associated with the history of allergic diseases and reversible lung obstruction (Macdowell and Peters, 2007; Monteseirin et al., 2002; Virchow, 2016). Allergens contact with the airway epithelium, disrupt its integrity as a physical barrier and trigger the release of alarmins including cytokines, uric acid, adenosine triphosphate, colony-stimulating factors and other substances (Hoffmann et al., 2016; Lambrecht et al., 2017). These agents activate and recruit the innate immune cells (dendritic cells, granulocytes, monocytes) from the bloodstream, ultimately leading to inflammation in the lungs (Kim et al., 2016; Lambrecht et al., 2017; Thiriou et al., 2017; Virchow, 2016). The severity of asthma affects the functioning of peripheral blood cells: in severe forms the number of granulocytes is significantly increased in the blood and bronchoalveolar lavage, and the expression profile of proinflammatory cytokines is also altered (Agache and Akdis, 2016; Brightling et al., 2008; Ciepiela et al., 2015; Nakagome et al., 2012; Shannon et al., 2008; Thiriou et al., 2017). Peripheral blood

Abbreviations: a.u, Arbitrary units; AUC, area under the curve; BA, bronchial asthma; CL, chemiluminescence; CI, confidence interval; CR3, CR4, complement receptors 3 and 4; EC_{50}, half maximal effective concentration; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; fMLF, peptide N-formyl-Met-Leu-Phe; FPR, formyl peptide receptor; GINA, Global Initiative for Asthma; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IgE, immunoglobulin E; LFA-1, lymphocyte function-associated antigen 1; OZ, opsonized zymosan; PEF, peak expiratory flow; PMA, phorbol 12-myristate 13-acetate; ROC, receiver operating characteristic; ROS, reactive oxygen species; TNF-α, necrosis factor-α; WKYMVM, peptide Trp-Lys-Tyr-Met-Val-D-Met-NH_2

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http://dx.doi.org/10.1016/j.imbio.2017.10.020
Received 29 July 2017; Received in revised form 20 September 2017; Accepted 4 October 2017

granulocytes in BA patients have a higher metabolic response and sensitivity to bacterial or viral agents compared to healthy donor cells (Asman et al., 1997; Tang et al., 2015; Tang et al., 2016; Ramirez-Velazquez et al., 2014; Vargas et al., 1998). Excess granulocyte activity is considered a factor of complications and exacerbation in asthma (Fahy, 2009).

Granulocytes circulating in blood are in a resting state, they can be physiologically primed (preactivated), activated, inactivated, and can die by apoptosis (El-Benna et al., 2016). Priming of granulocytes is particularly associated with an enhanced functionality of adhesion-associated responses that facilitates the recruitment of the cells from the blood to the airways (Kanters et al., 2007). Physiologically primed neutrophils was supposed can be deprimed in lung *in vivo* and return back in the pulmonary circulation as resting neutrophils (Singh et al., 2012; Summers et al., 2014). Primed neutrophil phenotype has been identified in humans with various inflammatory diseases, such as rheumatoid arthritis, chronic kidney disease, traumatic injury, and acute respiratory distress syndrome (Miralda et al., 2017). Not much is known regarding priming of inflammatory cells in an allergic asthma (Kanters et al., 2007; Luijk et al., 2005). Distinct priming profiles are associated with different phases, phenotypes, or both of allergic asthma (Kanters et al., 2007). Literature analysis showed that little attention is paid the state of the blood granulocytes in BA out of exacerbation.

Reactive oxygen species (ROS) play a key role in asthma pathogenesis due to accumulation and activation of granulocytes in the lungs (Al-Harbi et al., 2015; Jiang et al., 2014; Nadeem et al., 2014; Zuo et al., 2013). Excessive ROS production contributes to the development of oxidative stress, which leads to bronchospastic syndrome and the transition of the inflammatory process to a chronic form (Qu et al., 2017; Zanatta et al., 2012). However, a clear relationship between the ROS-producing activity of the blood cells and the disease severity is not currently established. Teramoto et al. (1996) found increased spontaneous and stimulated production of superoxide per an individual neutrophil in the whole blood of BA patients using a lucigenin-dependent chemiluminescence (CL). Hyperactivated phenotype with decreased (Mattheyse et al., 2001) or increased ROS production (Vargas et al., 1998) was detected during the acute BA stage using luminol-dependent CL. Also increased production of superoxide anion was recorded on reduction of cyt *c* in BA (Kanazawa et al., 1991). During exacerbations, even without stimulation granulocytes of BA patients had an increased ROS-generating response versus controls and patients without exacerbation. Contradictory data were obtained also in the remission period: without alterations in ROS level, as well as either increased or decreased ROS compared with healthy donors using luminol-dependent CL (Mattheyse et al., 2001; Monteseirin et al., 1996; Vargas et al., 1998). The isolated neutrophils of BA patients demonstrated increased ROS production in response to different stimuli estimated by various methods: reduction of cyt *c* (Meltzer et al., 1989; Styrt et al., 1988), nitroblue tetrazolium test (Mosca et al., 2015), lucigenin-dependent CL (Teramoto et al., 1996). Given that divergent results can be a consequence of the use of different methods for measuring ROS level (Caldefie-Chézet et al., 2002; Freitas et al., 2009; Monteseirín and Vega, 2011). The development of tests for monitoring the state of the blood cells is a difficult task. Serum ROS level was proposed to be a biomarker for predicting severe BA exacerbations. It was tightly associated with the degree of airway obstruction, neutrophil count and severe exacerbation (Nakamoto et al., 2016).

The granulocytes are the main effector cells in inflammation: they receive an alarm signal, leave the vascular bed and migrate to the inflammatory site along the chemotactic gradient of inflammatory factors. Adhesion interactions accompany different granulocytes' functions: diapedesis, migration through intercellular matrix to inflammatory centre, phagocytosis (Langereis, 2013). The adhesion to the endothelium, intracellular matrix and microorganisms is provided mainly by the expression of β2-integrins including $\alpha_M\beta_2$ (MAC-1, or CR3, or CD18/CD11b), $\alpha_L\beta_2$ (CD18/CD11a or LFA-1) and $\alpha_X\beta_2$ (CD18/CD11c, or CR4, or gp150/95). FcγR and CR3 receptors expressed on the cell membrane mediate the binding of opsonized particles and participate themselves in activation of ROS production (Nguyen et al., 2017). Opsonins are considered as molecules of antibodies IgM and IgG classes, fragments of complement components C3b, C4b, C-reactive protein and others. Neutrophils from patients with severe BA are known to express much more adhesion molecules than healthy donor cells (Aroca et al., 2014; Mukhopadhyay et al., 2014). Increased adhesion of peripheral blood eosinophils to E-selectin and ICAM-1 and neutrophils to ICAM-1 was observed in BA patients with a high degree of PEF variability. It was most probably caused by a functional upregulation of the receptors CDllb/CD18 and CD49d, because the number of receptors per cell was unchanged (Hakansson et al., 1995). The level of the soluble form of ICAM-1 serves as a marker of BA severity and is considered as a therapeutic target for asthma control (Mukhopadhyay et al., 2014).

Chronic inflammatory process underlies BA and a risk of frequent exacerbations exists, despite the permanent use of medications by patients (Hinks et al., 2016; Koczulla et al., 2017). So, simple and quick methods are necessary for monitoring the state of BA patients to personalize treatment and to prevent exacerbations. In this work we suggest testing the reactivity of the blood phagocytes in BA based of the time-course of respiratory response to opsonized zymosan (OZ) in the whole blood using luminol-dependent CL, as a measure of ROS production, and its activation kinetics. The fluorimetric, chemiluminometric and colorimetric probes were elaborated to identify and quantify ROS formed during the oxidative (respiratory) burst (Freitas et al., 2009). Many authors prefer to use luminol-dependent CL to detect ROS what is explained its advantage as following: the method has a high sensitivity; membrane-permeability of luminol allows to reflect the extra- and intracellular ROS unlike isoluminol detecting only extracellular ROS; luminol detects many oxygen intermediates like superoxide anion radical, hydrogen peroxide, hydroxyl radical, hypochlorite (Allen, 2015; Bedouhène et al., 2017; Dahlgren et al., 2008; Freitas et al., 2009; Monteseirín and Vega, 2011; Vladimirov and Proskurnina, 2009). Also nitric oxide produced by NO-synthase contributes luminol CL (Wang et al., 1993). All these oxygen spices were revealed in phagocytosis (Segal, 2005). The whole blood was more appropriate for our task to study cell reactions in experimental conditions which are maximal closed to native since the serum components, the products of phagocytes themselves and other cells can influence the phagocyte responses in complex. Microbe particles, e.g. zymosan, covered with serum opsonins, among which molecules of antibodies IgM and IgG classes, fragments of complement components C3b, C4b, C-reactive protein and others, are the experimental model of pathophysiological impact. When the microbial targets are opsonized, they are recognized more effectively by phagocytic receptors. Phagocytosis, physiological process critical in inflammation, initiates rather quick ROS production after recognition and internalization of opsonized microbes by the professional phagocytes (Cheson and Morris, 1981; Lim et al., 2017; Segal, 2005). Analysis of CL kinetics (K_s – the apparent Michaelis constant; V_{max} – the maximum velocity; T_n – the nodal time) revealed that luminol detects a relatively early event in OZ phagocytosis unlike lucigenin (Allen, 1986). The kinetic parameters of CL curves, which were recorded in the whole blood during phagocytosis of OZ, have shown the high discriminative possibilities in different diseases. The integral CL response and the time of reach half maximal value were useful indicators of CL defects in recurrent infections, myeloperoxidase deficiency, phagocytic defects and other (Carulli et al., 1995). Magrisso et al. (1995, 2000) presented OZ-initiated CL responses of isolated neutrophils and phagocytes in the blood as a sum of three biological components: (the first) processes that take place near the plasma membrane, connected with phagocytosis, cause extracellular CL; (the second) processes located inside the cell, connected with phagocytosis, and which cause intracellular CL; (the third) processes that lead to intracellular CL, not connected with phagocytosis. On the basis of the conception of three components of the CL curves the discriminative