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Technical Note

Ex vivo simulation of leukocyte function: Stimulation of specific subset of leukocytes in whole blood followed by the measurement of function-associated mRNAs

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ABSTRACT

In order to characterize a wide spectrum of leukocyte functions with clinically applicable procedures, 0.06 ml each of heparinized whole blood was stimulated in triplicate for 4 h with phytohemagglutinin (T cell stimulator), heat aggregated IgG (IgG Fc receptor stimulator), lipopolysaccharide (toll-like receptor (TLR)-4 stimulator), zymosan (TLR-2 stimulator), monoclonal antibody against T-cell receptor alpha/beta chain, recombinant interleukin-2, and solvent controls, then 32 different leukocyte function-associated mRNAs were quantified by the method reported previously (Mitsuhashi et al. Clin. Chem. 2006). Two control genes (beta-actin, beta-2-microglobulin) were not affected by these stimulations, whereas the induction of CCL chemokines-2, 4, 8, 20, CXCL chemokines-3, 10, interleukin (IL)-8 (markers of leukocyte accumulation/recruit), granzyme B, perforin 1, tumor necrosis factor superfamily-1, 2, 5, 14, 15, CD16 (markers of cell killing), IL10, transforming growth factor beta 1 (humoral factors of immune suppression), forkhead box P3, CD25, arginase (cellular markers of immune suppression), IL2, IL4, interferon-gamma, IL17 (markers of various subsets of T helper cells), granulocyte-macrophage colony-stimulating factor (marker of antigen presenting cells), immunoglobulin heavy locus (marker of B-cells), vascular endothelial growth factor (marker of angiogenesis), pro-opiomelanocortin (marker of local pain), and CD11a mRNA (marker of leukocyte adherence to endothelium) were identified by these stimulations. The blood volume in this assay was 1.44 ml, and 4 h' incubation in whole blood was physiological. Using triplicate aliquots of whole blood for both stimulant and solvent control, statistical conclusion was drawn for each stimulant for each mRNA. The method introduced in this study will be a new paradigm for clinical cellular immunology.

1. Introduction

Leukocytes circulating in peripheral blood are generally in a steady state, and once they migrate to local lesions of inflammation, neoplasms, foreign bodies (microorganisms, transplanted tissues and devices, drugs and vaccines), etc., they are fully activated in a specific way dependent on the type of leukocytes and the type of local stimulants. In order to simulate such leukocyte responses, crude whole blood was exposed to various specific and general stimulants and each leukocyte response was quantified in this study.

Traditionally, leukocytes are isolated and cultured with or without specific stimuli for a couple of days to several weeks to

Abbreviations: ATP, adenosine-5'-triphosphate;ELISA, enzyme-linked immunosorbent assay;PCR, polymerase chain reaction;TCR, T cell receptor;HAG, heat aggregated IgG;PHA, phytohemagglutinin;LPS, lipopolysaccharide;rIL2, recombinant interleukin 2;PBS, phosphate buffered saline;Ct, cycle threshold; ABI, Applied Biosystems;ACTB, β-actin;B2M, β2-microglobulin;GZB, granzyme B;PRF1, perforin 1;TNFSF, tumor necrosis factor superfamily;IL, interleukin; IFNG, interferon-γ;GMCSF, granulocyte-macrophage colony-stimulating factor; TGFB1, transforming growth factor beta 1;FOXP3, forkhead box P3;IGH@, immunoglobulin heavy locus;ARG1, arginase;VEGF, vascular endothelial growth factor;POMC, pro-opiomelanocortin;FcγR, IgG Fc receptor;ADCC, antibody-dependent cell-mediated cytotoxicity;NK, natural killer;TLR, toll-like receptor; Treg, regulatory T-cells;MDSC, myeloid derived suppressor cells.

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identify the functional changes (protein synthesis and secretion, apoptosis, cell proliferation, surface marker changes, etc.). However, due to the complexity of the assay, this is not generally applicable as a routine diagnostic test. To overcome this technical difficulty associated with cell isolation and culture condition, attempts were made to use whole blood with short incubation (typically overnight) with specific stimuli, and quantify the amount of adenosine-5'-triphosphate (ATP) (Cylex's ImmuKnow) or measure the levels of various cytokines by enzyme-linked immunosorbent assay (ELISA). However, the use of ATP is limited, and is not applicable to a wide variety of leukocyte functions. The detection limit of typical ELISA is picomole to femtomole (10^{11} to 10^{9} molecules), which is far less sensitive than single molecule detection by polymerase chain reaction (PCR).

In the present study, we focused on the measurement of *ex vivo* induction of leukocyte-function-associated mRNAs, because mRNA induction happens much earlier than protein synthesis and corresponding biological changes. The use of whole blood allows complex cell-to-cell communication and interaction with plasma factors and proteins. Although mRNA analysis in whole blood is well established and FDA-approved product is commercially available (PAXgene), this is a snapshot of gene expression at the time of blood draw (Landes et al., 2008). In contrast, the present study analyzed the fluctuation of the levels of mRNA after appropriate stimulation. This is like a video clip compared to the snapshot.

Although PCR is sensitive enough to detect a single copy of target gene, the sensitivity of *ex vivo* study is dictated by the variation among triplicate aliquots of whole blood. The variation will be induced at any step from leukocyte isolation, RNA purification, cDNA synthesis, to PCR, and tiny variation introduced at any step will be enhanced exponentially during PCR. Moreover, even from a single blood sample, many aliquots are generated based on the number of stimulants, dose responses, time course, combinations of stimulants, duplicate or triplicate, etc. Thus, high throughput assay platform is desirable. Due to these strict requirements, this study used the assay platform introduced previously (Mitsuhashi et al., 2006, 2008a,b;Mitsuhashi and Targan, 2008).

2. Materials and methods

2.1. Materials

Anti-T cell receptor α/β chain (TCR) monoclonal antibody (IgG1 κ) and control mouse IgG1 κ were obtained from BioLegend (San Diego, CA, USA). Reverse transcriptase, dNTP, and RNasin were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Immune complex (heat aggregated IgG, HAG) was prepared by heating human IgG at 63 °C for 15 min as described previously (Ostreiko et al., 1987). In 8-well strip microtubes, 1.2 μl each of phytohemagglutinin-L (2 mg/ml), HAG (10 mg/ml), lipopolysaccharide (LPS) (0.5 mg/ml), zymosan A (75 mg/ml), recombinant interleukin 2 (rIL2) (5 μg/ml), phosphate buffered saline (PBS), anti-TCR antibody (50 μg/ml), and control IgG (50 μg/ml), were added respectively into 8 wells, and stored at −80 °C until use.

2.2. Blood treatment

Heparinized whole blood samples were obtained from Apex Research Institute (Tustin, CA, USA) after Institutional Review Board approvals. In order to equalize post-blood collection condition, blood samples were stored at 4 °C overnight. Next morning, blood was decanted into a reservoir (Fig. 1A), and using 8-well multi-channel pipette, 60 μl each of blood was dispensed into 3 strips. The blood volume needed for this test was 1.44 ml (= 60 μl/well × 8 wells × 3 strips (triplicate)). After cap was closed, strips were incubated at 37 °C for 4 h, then stored frozen at −80 °C.

2.3. Target mRNAs

The mRNA sequences of target mRNAs were retrieved from GenBank. PCR primers were designed within the coding region by Primer Express (Applied Biosystems (ABI), Foster City, CA, USA) (Supplemental Table 1). Oligonucleotides were synthesized by IDT (Coralville, IA, USA). The target mRNAs (total 32) were β-actin (ACTB), β2-microglobulin (B2M), granzyme B (GZB), perforin 1 (PRF1), tumor necrosis factor superfamily (TNFSF)-1, 2, 5, 14, and 15, CCL chemokines-2, 4, 8, and 20, CXCL chemokines-3, and 10, interleukin (IL)-2, 4, 6, 8, 10, and 17A, interferon-γ (IFNG), granulocyte-macrophage colony-stimulating factor (GMCSF), CD11a, 16 and 25, transforming growth factor beta 1 (TGFB1), forkhead box P3 (FOXP3), immunoglobulin heavy locus (IGH@), arginase (ARG1), vascular endothelial growth factor (VEGF), and pro-opiomelanocortin (POMC) (Table 1).

2.4. mRNA analysis

Fifty μl of frozen-thaw blood was applied to 96-well custom filterplates, and leukocytes were isolated on the filter membranes by centrifugation (Fig. 1B) (Mitsuhashi et al., 2006). Sixty μL of lysis buffer containing a cocktail of specific reverse primers was applied to the filterplates, and the resultant cell lysates were transferred to oligo(dT)-immobilized microplates (Hamaguchi et al., 1998) for poly(A)$^{+}$ mRNA purification (Fig. 1C). The cDNA was directly synthesized in 50 μl solutions at each well: specific primer-primed cDNA in the liquid phase and oligo(dT)-primed cDNA in the solid phase (Mitsuhashi et al., 2006). The liquid and solid phase cDNAs were used for real time PCR using iTaqSYBR (Biorad, Hercules, CA, USA) (Morrison et al., 1998) in thermal cyclers (PRISM 7900, ABI, and iCycler, Biorad, respectively) (Fig. 1D). PCR condition was 95 °C for 10 min followed by 50 cycles of 65 °C for 1 min and 95 °C for 30 s. For IL2 and IL4, 4 μl of undiluted cDNA solution was used in the final volume of 10 μl in 384 well PCR plate. For FOXP3, ARG1, IFNG, GMCSF, and POMC, 2 μl of undiluted cDNA solution was used for PCR in the final volume of 5 μl. The cDNA was then diluted 1:2 by adding 32 μl of DNase/RNase free water, and 2 μl each of cDNA was used for PCR for remaining 24 genes (except 1L17A) in the final volume of 5 μl. After the leftover cDNA was transferred to fresh strip microtubes, solid phase cDNA was used directly to amplify IL17A by adding 30 μl PCR solution. The melting curve was always analyzed to confirm that PCR signals were derived from a single PCR product. The cycle threshold (Ct) was determined by analytical software