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Research paper

The pre-analytical processing of blood samples for detecting biomarkers on protein microarrays

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ABSTRACT

Specimen collection method and quality insurance are pivotal in biomarker discovery. Pre-analytical variables concerning blood collection and sample handling might affect analytical results and should be standardised prior application. In this study, we examine pre-analytical characteristics of blood samples using protein microarray. The influences of 1) standby times until centrifugation (1 h, 4 h, 24 h and 48 h), 2) four blood collection methods, and 3) IgG purified from those samples on differentially reactive antigens between samples ("DIRAGs") were investigated. Spearman correlation analyses of intra-individual arrays demonstrated remarkable differences (0.75–0.98 vs. 0.5–0.75) of antibody reactivities within and between serum and plasma samples. Class comparison showed that reactive antigen profiles were best preserved using IgG purified samples of serum tubes without separation gel as after 24 h 83% of the 1 h baseline DIRAGs were re-found. Testing dilution series with protein microarrays and Luminex xMap® Technology, we found linear correlations ($R^2 = 0.94–0.99$) between IgG concentration and read-out when using purified IgG instead of serum. Therefore, we highly recommend standardising pre-analytics and proposing the use of purified IgG for autoantibody immune-profiling with protein microarrays to reduce potential unspecific binding of matrix proteins abundant in serum and plasma samples.
Significance: Although purified IgG cannot completely compensate the influence of pre-analytics, in highly parallel immune-profiling IgG enables reduction of unspecific effects, which occur when using serum or plasma for analysis on protein microarrays. Reproducibility problems due to pre-analytical processing of blood samples might explain discrepant results reported in various biomarker studies.

1. Introduction

Specimen collection is a crucial component in the clinical diagnostics as well as in biomarker discovery. Samples from diseased patients at various stages and control sera from healthy individuals are key components of analysing biomarkers. In general, biomarkers provide valuable information about normal biological or pathological processes or about pharmacologic response to therapeutic interventions (Murphy et al., 2012). Depending on the site of evaluation, biomarkers can be categorized into tissue and circulating biomarkers (Ullah and Aatif, 2009), both groups include DNA, RNA and protein molecules. As the main working machinery in the cell, proteins truly reflect the physiological state of cell activities, making them more suited as biomarkers than the DNA itself (Griffiths, 2007). Autoantibodies that target specific tumour-associated antigens (TAAs) are formed at an early stage during

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tumourigenesis, which might be months or years before the clinical diagnosis of cancer (Luna Coronell et al., 2012). Furthermore, they play a pathogenic role in autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis or scleroderma (Pollard, 2006; Shoenfeld and Gershwin, 2000). Since only few microlitres of patient's blood or serum is necessary for its detection autoantibodies became highly attractive biomarker candidates for early diagnostic and minimal invasive testing (Tan et al., 2009).

Protein microarray technology (Griffiths, 2007; Gunawardana and Diamandis, 2007; Lu et al., 2008) is an innovative and highly multiplexed platform for the identification of autoantibodies using immobilised antigens. The method has been applied to identify autoantibody markers in prostate (Massoner et al., 2012), brain, lung (Stempfer et al., 2010), colon (Babel et al., 2009) and breast cancers (Anderson et al., 2008, 2011; Syed et al., 2012a, Syed et al. 2012b). Sensitivities in the range of 44–95% and specificities in the range of 80–100% have been reported in several promising studies (Luna Coronell et al., 2012).

However, a critical problem in the context of biomarker detection is the lack of standardisation concerning the collection and quality of blood-derived serum and/or plasma samples (Bracci et al., 2012; Luna Coronell et al., 2012). Therefore, pre-analytical variables concerning blood collection and sample handling should be investigated and standardised (Lippi et al., 2006; Zhao et al., 2012). It has been reported that factors, such as anticoagulants (heparin, EDTA salts, citrate and fluoride) or gels in blood collection tubes, sample processing temperatures and time until centrifugation, sample haemolysis, storage conditions and the number of freezing and thawing-cycles might affect the analytical results (Tuck et al., 2009). A major problem in this context is the comparability among different biomarker studies, since specimens used are often collected and handled in completely different ways.

The two common sampling materials used for immunological analyses are serum and/or plasma. In general, blood collection procedure involves sample drawing, clotting, centrifugation and separation of serum or plasma from the clot and/or cells, transfer and storage. The collection tubes for serum may contain clot activators like thrombin and sometimes gels to separate the blood clot from the serum. The collection tubes for plasma usually contain anticoagulants such as heparin, EDTA salts or citrate. In general, plasma contains a considerable amount of highly abundant proteins (such as albumin, transferrin, fibrinogen and complement factors), which constitute 97–99% of the total protein content of blood, and hundreds of diverse proteins, which represent the remaining 1% (Correia, 2010). Though it has been estimated that serum contains 3–4% less protein than plasma (Correia, 2010), both blood sample specimens contain about 7–16 g/L immunoglobulin G, in short IgG (Dati et al., 1996).

Immunoglobulin G is the most abundant circulating antibody isotype and represents about 75% of serum immunoglobulins in humans (Junqueira and Carneiro, 2005). The molecular weight of the IgG monomer is about 150 kDa, consisting of two identical heavy and two identical light chains connected by disulphide bridges. The IgG class contains four isotypes (IgG1, IgG2, IgG3 and IgG4), which show different features regarding half-life, stability, serum concentration and immune mechanism (Correia, 2010). The purification methods include immune-precipitation (Firestone and Winguth, 1990) and column purification with proteins A, G, A/G and L or new approaches such as Melon™ Gel (Pierce Biotechnology Inc., Rockford, IL, USA), thiophilic adsorbent, mannan-binding protein and immune-affinity chromatography using a specific immobilised antigen. About 10 billion different IgG antibody moieties circulate in a healthy adult and those are in a constant flux, therefore it is quite a challenge to identify the disease specific low abundance IgGs (Stafford and Johnston, 2011). Thus for biomarker development, efforts have to be made to select an appropriate methodology to maintain the antibody diversity and avidity in the samples.

In this paper, we describe the effect of different blood collection methods and specimen handling times (1 h up to 48 h) on antibody profiles between different donors using serum and plasma samples, as well as purified IgG. For the sake of simplicity the statistically significant, differentially reactive antigens between two individuals or samples here we abbreviated to DIRAGs. Blood of four healthy female donors was collected in plasma tubes with EDTA and serum tubes with or without separation gel using different standby times before centrifugation. Serum, plasma and IgG purified samples were analysed on protein microarrays. Finally, the effects of different conditions on antibody diversity were determined. Here we report that pre-analytical processing of blood samples using serum tubes without separation gel and purified IgG best maintains antibody diversity in the samples and ensures linearity measurements. Therefore we propose the application of purified IgG as a specimen for detecting TAAs on protein microarrays.

2. Materials and methods

2.1. Blood samples

Blood samples were obtained after consent was given from laboratory staff (four healthy females encodes as 1, 2, 3 and 4) of the Medical University of Vienna and the General Hospital of Vienna. Four different collection tubes were analysed using Greiner Bio One (Kremsmünster, Austria) collection tubes including serum tubes with separation gel (SG samples), serum tubes without separation gel (SW samples), EDTA tubes for plasma (PE samples) and EDTA tubes for plasma with subsequent density gradient centrifugation (PD samples). The different time lags until centrifugation of the blood samples at room temperature were 1 h, 4 h, 24 h and 48 h after sampling. The density gradient centrifugation of the PD samples had been performed according to Brandt and Griwatz (1996). Saturated (1% FCS in PBS) polypropylene conical tubes containing 3 mL PolymorphPrep™ (d = 1.113 g/mL, osmolality = 460 mOsm; PROGEN Biotechnik GmbH, Heidelberg, Germany) were carefully overlayed with 3 mL NycoPrep™ (d = 1.068 g/mL, osmolality = 335 mOsm; PROGEN Biotechnik GmbH). Five millilitre blood of an EDTA-tube was layered on top of the gradient-tubes and after centrifugation the clear plasma fraction was collected from the top. All other tubes were handled according to the manufacturer's instructions. Samples were stored at −80 °C until protein microarray analyses.

2.2. IgG purification of blood samples

Purification of immunoglobulin G from all samples was performed according to the manufacturer's instructions of the