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Research paper

Selective, sensitive and comprehensive detection of immune complex antigens by immune complexome analysis with papain-digestion and elution

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ABSTRACT

Comprehensive identification and profiling of antigens in immune complexes (ICs) in biological fluids, such as serum and cerebrospinal fluid, is useful for developing early diagnostic markers and specific treatments for many diseases. We have developed a method, designated "immune complexome analysis", to comprehensively identify the antigens in ICs. In this method, we first purify ICs from biological fluid by using Protein G- or Protein A-coated beads, then these ICs are subjected to tryptic digestion on the beads and subsequent analysis using nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS). We previously used this method to find specific antigens in circulating ICs (CIC-antigens) in serum for autoimmune diseases, infectious disease and cancers. However, this method detects not only CIC-antigens but also antibodies and proteins bound non-specifically to the beads, which restricts the detection of minor peptides released by the digestion of CIC-antigens whose amounts are generally much less than antibodies and the proteins. To selectively detect CIC-antigens with enhanced sensitivity, in this study we compared three methods (Method A, direct tryptic digestion on the beads; Method B, low-pH elution and tryptic digestion; Method C, papain-digestion, elution, and tryptic digestion) and examined which method selectively elutes CIC-antigens from CICs bound to the beads and selectively detects CIC-antigens using nano-LC-MS/MS. We also compared three types of CIC-capturing beads (Protein G-coated magnetic beads, Protein A-coated magnetic beads and Proceptor™-sepharose beads) to examine if parallel use of these beads aids the comprehensive detection of CIC-antigens in immune complexome analysis. Comparison showed that Method C provided the most selective and sensitive detection of CIC-antigens, without interference by antibodies and proteins non-specifically bound to the beads. In addition, using three types of beads allowed the examination of a wide range of CIC-antigens in immune complexome analysis. Therefore, combining Method C with three types of beads should allow the selective and sensitive identification of IC-antigens present in biological fluids from patients with a variety of diseases. The identification of IC-antigens may lead to the development of diagnostic methods and protocols for specific treatments for these diseases.

1. Introduction

Immune complexes (ICs) are formed upon noncovalent interaction between foreign antigens or autoantigens and antibody proteins (Nezlin, 2000). Enhanced formation and defective clearance of ICs occurs in autoimmune diseases, cancers, and infections, which triggers such diseases (Chauhan, 2017). Therefore, comprehensive identification and profiling of antigens present in ICs is useful to find targets for developing early diagnostic markers and specific treatments for these diseases. To validate this concept, we have developed a method, designated "immune complexome analysis", to catalogue antigens in ICs. In this approach, ICs are isolated from biological fluids, such as serum and cerebrospinal fluid, by using Protein G- or Protein A-coated beads that bind the fragment of crystallization (Fc) domain of antibodies, and the ICs are then subjected to tryptic digestion (in which the ICs are directly digested on the beads without eluting them) and analyzed using nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) (Ohyama et al., 2011). We have successfully used this

Abbreviations: CIC, circulating immune complex; IC, Immune complex; Ig, immunoglobulin; nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry

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method to identify specific antigens in circulating ICs (CIC-antigens) in serum or cerebrospinal fluid for autoimmune diseases (Ohyama et al., 2011, 2012, 2015a, 2015b; Aibara et al., 2018a), infectious disease (Ohyama et al., 2016), cancers (Ohyama et al., 2017) and liver transplant recipient (Aibara et al., 2018b).

However, our own work and work from other laboratories has demonstrated remarkable interference from abundant proteins and antibodies in identifying antigens from CICs (Trinkle-Mulcahy et al., 2008). In our method, not only CICs but also large amounts of antibodies and proteins that bind non-specifically to beads are detected. Large amounts of peptides from these dominant proteins make the detection of minor peptides from CIC-antigens difficult, resulting in a low number of identified CIC-antigens and incorrect profiling of CIC-antigens by immune complexome analysis. To enhance the selectivity and sensitive detection of CIC-antigens by immune complexome analysis, CICs should be selectively eluted from the beads and then digested prior to MS/MS analysis.

We examined two methods for the selective elution of antigens from CICs on the beads: low-pH elution and papain-digestion elution. Low-pH elution, in which solvent polarity as well as electrostatic and hydrophobic interactions between antigens and antibodies are reduced by using a low pH eluent, is the most frequently used method to separate antigens and antibodies (Yarmush et al., 1992; Li et al., 2007; Gustaw et al., 2008; Kavita et al., 2017). On the other hand, papain selectively cleaves immunoglobulin (Ig) at the heavy chain hinge region into three fragments: one Fc and two identical Fab fragments (Bennett et al., 1997; Moorhouse et al., 1997; Adamczyk et al., 2000). Therefore, we envisioned that papain would cleave antibodies in CICs at the hinge region, followed by selective dissociation of the antigens from CICs without eluting the proteins bound non-specifically to the beads. In this study, we thus compared three methods: Method A, direct tryptic digestion on the beads; Method B, low-pH elution and tryptic digestion; Method C, papain-digestion, elution, and tryptic digestion (Fig. 1). The methods were applied to the analysis of a serum sample as well as an *in vitro* formed immune complex (complex of myoglobin and anti-myoglobin) to examine which method selectively elutes antigens from CICs bound to the beads and allows the sensitive detection of antigens using nano-LC-MS/MS.

We also compared three types of beads (Protein G-coated magnetic beads, Protein A-coated beads, and Proceptor™-sepharose beads) used for CIC isolation. We investigated the profiles of CIC-antigens using each bead type and assessed whether parallel use of these beads aids the comprehensive detection of CIC-antigens in immune complexome analysis.

2. Materials and methods

2.1. Materials and chemicals

Magnetic beads with immobilized Protein G or Protein A were purchased from Millipore (Billerica, WI, USA). Proceptor™-sepharose beads were purchased from ProGen Biologics (Wildwood, MO, USA). Equine myoglobin was from Sigma (St. Louis, MO, USA) and goat anti-myoglobin antibody was purchased from Bethyl Laboratories (Montgomery, TX, USA). Human pooled serum was from Funakoshi (Tokyo, Japan). Dithiothreitol, formic acid, ultrapure water, acetonitrile, acetic acid, and phosphate-buffered saline (PBS, 9.0 mmol/L Na_2HPO_4, 2.9 mmol/L NaH_2PO_4, 137 mmol/L NaCl, pH 7.4) (all high-performance liquid chromatography (HPLC) grade), ethylenediaminetetraacetic acid (EDTA) and glycine were obtained from Wako (Osaka, Japan). Iodoacetamide was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ammonium hydrogen carbonate, trifluoroacetic acid (TFA), tris (hydroxymethyl)aminomethane, hydrochloric acid and L-cysteine were obtained from Nacalai Tesque (Kyoto, Japan). Papain was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Trypsin was obtained from Promega (Madison, WI, USA).

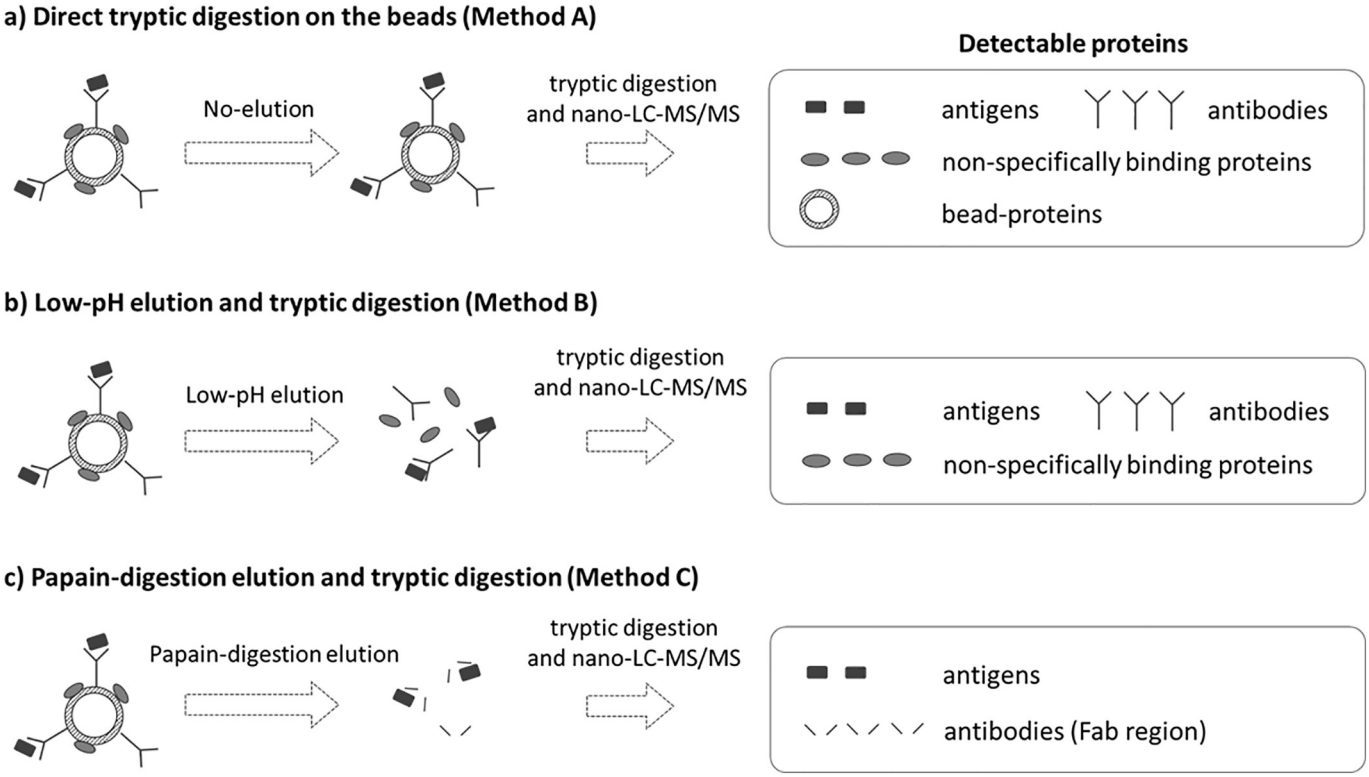

Fig. 1. Proteins likely detected by the three protocols. a) Direct tryptic digestion on the beads, b) Low-pH elution and tryptic digestion, c) Papain-digestion, elution, and tryptic digestion.