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Technical note

A method for extracting tissue proteins for use in lymphocyte function assays

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ABSTRACT

Currently human T-cell responses to unpurified tissue extracts cannot be easily measured because current cell lysis methods yield a lysate that is toxic. Here we describe the optimization of a protocol for extracting proteins from human tissues in a format that is compatible with the functional analysis of human T cells. The tissue was homogenized in a mixture of butan-1-ol, acetonitrile and water and then lyophilized. Lyophilized protein extracts were dissolved in 8 M urea because urea did not affect T-cell function when present at 0.08 M or less. Using this method cytokine production and proliferation responses were detected from islet, acinar and spleen extracts. Hence, our method allows the rapid preparation of human tissue lysates in a format that is compatible with the analysis T-cell responses. We suggest that this method will facilitate the analysis of adaptive immune responses to tissues in transplantation, cancer and autoimmunity.

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1. Introduction

Measuring T-lymphocyte responses to antigens is central to the study of adaptive immune responses. Over the years many assays for lymphocyte function, such as ELISA, ELIspot, intracellular cytokine staining, ^{3}H-thymidine incorporation and CFSE-based proliferation assays have been developed (for a review see (Mannering and Brodnicki, 2007)). While these techniques allow the function of antigen-specific T cells to be analyzed the quality of the results depends upon the purity of the antigen and its fidelity to native protein. Analysis of adaptive immune responses against tumor antigens in cancers, or self-antigens in autoimmune diseases, has been more challenging. The responses to these antigens are invariably weak, due to the scarcity of antigen-specific T cells. For example, the quality of the antigen preparation has been reported to be a significant obstacle in the analysis of human T-cell responses to antigens recognized by T cells from people with in type 1 diabetes (Peakman et al., 2001; Mannering et al., 2003b).

Tissue extracts are a useful form of antigen for analyzing adaptive immune responses in cancer and autoimmune disease because no knowledge of the target antigen is required. Current methods for preparing tissue extracts use detergents. We found detergent-based methods for extracting tissue proteins to be unsuitable for assays that require viable cells because traces of detergent make the extract toxic to PBMC. Hence, we set out to develop a method for extracting protein from human tissue in a form that is compatible with functional T-cell assays.

2. Materials and methods

2.1. Protein extraction

Butan-1-ol, 2-methyl propanol, acetonitrile, and methanol (all from Sigma-Aldrich, St. Louis MO, USA) were diluted in

Abbreviations: CFSE, 5,6-carboxylfluorescein diacetate succinimidyl ester; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; TT, tetanus toxoid; CDI, cell division index; PHS, pooled human serum; BCA, bicinchoninic acid assay.

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distilled water and used at the concentrations indicated. Spleen and pancreatic islet tissue samples were obtained from cadaveric organ donors with informed consent from next of kin (St. Vincent's Hospital ethics committee approval protocol No. HREC-A 011/04). Islets and acinar tissue were prepared from pancreata as described (Ricordi et al., 1989; Campbell et al., 2008). Small pieces of spleen (100–700 mg), islets and acinar tissue were snap frozen in liquid nitrogen and stored at −80 °C. To prepare tissue extracts, samples were homogenized in 3–10 ml of solvent solution and centrifuged at 2000 rpm for 10 min. The supernatant was collected and the concentration of protein in the supernatant was determined by Bicinchoninic Acid (BCA) Assay (Pierce, Thermo Fischer Scientific, Rockford, IL, USA). For optimization experiments the results are expressed as the total yield of protein as a percentage of the starting mass of tissue ((mass of protein recovered/mass of starting tissue)×100 = % recovery). Protein solutions were lyophilized overnight and the dry extract was dissolved in 8 M urea/PBS under gentle rotation for 1 h at 4 °C. Aliquots of 10 µl of tissue extract containing 100 µg of protein were dispensed into sterile 5 ml tubes (BD, Franklin Lakes, NJ, USA) and stored at −80 °C. Twelve percent Tris–HCL gels were run on a BioRad Protean II electrophoresis system (Gladesville, NSW, Australia) using a Laemmli buffer system. Tissue lysates were dissolved in 8 M urea/PBS and 10 µg of protein was mixed with an equal volume of Laemmli sample buffer, reduced with 2-mercaptoethanol and boiled for 2 min prior to loading. Protein bands were visualized by staining with 0.1% Coomassie Blue (BioRad, Gladesville, NSW, Australia) Molecular weight markers were SeeBlue pre-stained standards (Invitrogen, Carlsbad, CA, USA).

2.2. Testing solvents for compatibility with functional assays

The following chemicals were tested for toxicity against human PBMC: dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), 8 M urea and 6 M guanidine (both in PBS) (all from Sigma-Aldrich, St. Louis, MO, USA). Urea was dissolved in PBS overnight, pH adjusted to 7.5 and sterilized by filtration through a 0.22 µm filter (Millipore, Billerica, MA, USA). Each solvent was diluted in complete culture medium (RPMI-1640/5% pooled human serum) to the final concentrations indicated in Fig. 2. For anti-CD3 mAb (OKT3) driven proliferation, 1.0×10^5 PBMC were added to each well with OKT3 (10 ng/ml) and the indicated

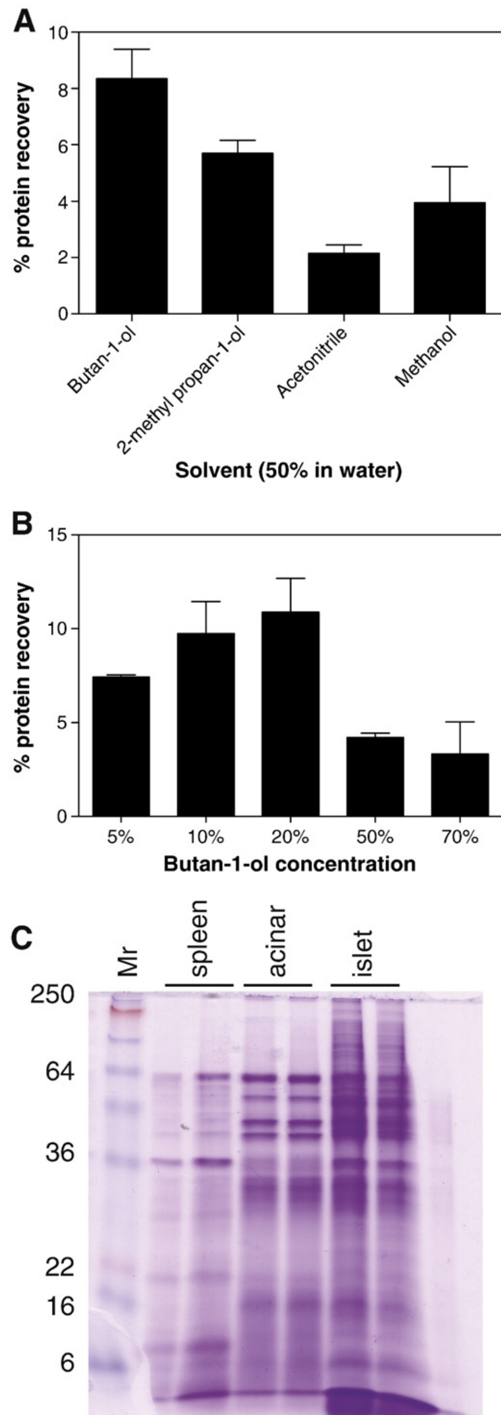

Fig. 1. Comparison of solvents for tissue extraction. Each solvent was mixed with an equal volume of water (A) and used to extract protein from a known mass of human spleen. The difference between butan-1-ol, 2-methyl propan-1-ol and methanol did not reach statistical significance ($p>0.05$). (B) Solvent mixtures were prepared with varying concentrations of butan-1-ol indicated a fixed concentration of acetonitrile (30%). The concentration of protein in each solvent solution was determined by BCA assay and the percentage protein recovery was calculated by dividing the total recovered protein by the starting weight of tissue, expressed as a percentage. The difference between 5%, 10% and 20% butan-1-ol did not reach statistical significance ($p>0.05$), but all gave significantly greater yields than 50% butan-1-ol ($p<0.033$). For both experiments; the bars represent the mean and standard error of the mean (SEM), of triplicate protein estimations, a representative of three experiments is shown. (C) Analysis of the tissue extracts by electrophoresis. Duplicate lanes containing 10 µg of each tissue extract, stained with Coomassie Blue are shown.

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