Methods in Free Radical Biology and Medicine

Detection and isolation of human serum autoantibodies that recognize oxidatively modified autoantigens

Paul Eggleton [a,b], Ahuva Nissim [c], Brent J. Ryan [d], Matthew Whiteman [a], Paul G. Winyard [a,*]

[a] University of Exeter Medical School, St Luke's Campus, Exeter, Devon EX1 2LU, UK
[b] Department of Biochemistry, University of Alberta, Edmonton, AB, Canada T6G 2H7
[c] Bone and Joint Research Unit, William Harvey Research Institute, Barts and The London, Queen Mary's School of Medicine and Dentistsry, University of London, London EC1M 6BQ, UK
[d] Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1 3QX, UK

A R T I C L E   I N F O

A B S T R A C T

The breakdown of human immune tolerance to self-proteins occurs by a number of mechanisms, including posttranslational modifications of host molecules by reactive oxygen, nitrogen, or chlorine species. This has led to great interest in detecting serum autoantibodies raised against small quantities of oxidatively modified host proteins in patients with autoimmune inflammatory diseases, such as rheumatoid arthritis. Here, we provide protocols for the preparation and chemical characterization of oxidatively modified protein antigens and procedures for their use in immunoblotting and ELISAs that detect autoantibodies against these antigens in clinical samples. These gel electrophoresis- and plate reader-based immunochemical methods sometimes suffer from low analytical specificity and/or sensitivity when used for serum autoantibody detection. This is often because a single solid-phase protein (antigen) is exposed to a complex mixture of serum proteins that undergo nonspecific binding. Therefore more sensitive/specific techniques are required to detect autoantibodies specifically directed against oxidatively modified proteins. To address this, we describe novel affinity chromatography protocols by which purified autoantibodies are isolated from small volumes (< 1 ml) of serum. We have also developed strategies to conjugate submilligram amounts of isolated immunoglobulins and other proteins to fluorophores. This set of methods will help facilitate the discovery of novel diagnostic autoantibodies in patients.

Introduction

Antibodies generated by B lymphocytes play a crucial role in the specific recognition, binding, neutralization, and ultimately

elimination of foreign pathogens from the human body [1]. In many autoimmune diseases (for example, rheumatoid arthritis (RA)[1], systemic lupus erythematosus (SLE), ankylosing spondylitis, psoriatic arthritis, and myositis) numerous autoantibodies are generated that react against host tissue and might promote disease pathology, particularly in rheumatological disorders [2]. Autoantibodies are also implicated in other disease processes such as atherogenesis (autoantibodies to oxidized low-density lipoprotein—oxLDL) [3,4] and lung inflammation (autoantibodies to protein carbonyls) [5]. Many autoantibodies are generated against abundant tissue antigens such as blood cells, deoxyribonucleic acid, extracellular matrix components, and plasma proteins. For example, in RA patients, a well-recognized antigen is the extracellular matrix protein of joint cartilage, type II collagen [6], and in SLE patients, autoantibodies against the plasma complement component, C1q, are well described [7]. This contradicts the commonly

accepted phenomenon of "immunological tolerance," whereby the body is prevented from mounting an immune response against itself.

All self-proteins/antigens (e.g., type II collagen fragments and C1q) are engulfed by antigen-presenting cells and presented on the surface of these cells, via major histocompatibility class II molecules, to T cells via T cell receptors or B cells via surface immunoglobulins. Normally the lymphocytes do not mount an immune response to these presented self- antigens (Fig. 1A). Early during embryonic development, the immune system maintains tolerance by either eliminating self-reactive cells or altering a cell's capacity to respond to self—a process called cell anergy. Some immune cells can be made to remain in a state of tolerance, through regulation by other cells. Other potentially autoreactive cells are excluded or restricted from sites at which an inflammatory immune response would have severe consequences in terms of loss of function (e.g., the brain and eyes). These effects are linked with the phenomenon of "immune privilege." Despite these safety mechanisms, in autoimmune diseases these self-antigens initiate an adaptive immune response through activation of autoreactive T cells that in turn promote the activation of B cells via cytokines to generate autoantibodies.

It is unclear what makes a self-antigen appear foreign to the immune system, but there is increasing evidence that the post-translational modification (PTM) of certain self-antigens can convert innocuous self-antigens into immunopathological triggers of autoantibody production, leading to cell death and a vicious cycle of inflammation [8]. Many different PTMs could be involved [9]—such as phosphorylation, proteolytic cleavage, ubiquitination, and SUMOylation—but oxidative modifications by reactive oxygen species (ROS) are attractive candidates. Such oxidative modification of self-antigens (e.g., type II collagen and C1q) might initiate a process of "oxidative PTM intolerance," resulting in a primary B cell response against the posttranslationally modified self-antigen. Additionally, a chronic secondary response may involve the development of autoreactive B cells that generate plasma cells. By a process of "epitope spreading" [10], autoreactive B cells may secrete autoantibodies against unmodified host proteins (Fig. 1B). Trinder and colleagues [11] proposed that this mechanism accounted for the generation of autoantibodies against common host proteins, such as anti-collagen antibodies in RA patients.

A wide variety of oxidative/nitrative PTMs might be involved in eliciting autoantibody formation [12,13]. Among the oxidation-susceptible amino acid side chains of proteins, the cysteine thiol (–SH) group may be oxidized to cysteine sulfenic, sulfinic, and sulfonic acid derivatives in the presence of H_2O_2. Cysteine can also be oxidized to form a disulfide (RS–SR) with another free thiol. Methionine is oxidized to methionine sulfoxide and further to methionine sulfone. Attack on phenylalanine by the hydroxyl radical ($^\bullet OH$) results in the hydroxylation of this aromatic amino acid, with the formation of *o*-tyrosine, *m*-tyrosine, and 3,4-dihydroxyphenylalanine. The $^\bullet OH$ radical also cross-links tyrosine, to form stable inter- or intramolecular protein cross-links. The action of ROS on tryptophan causes hydroxylation of the indole ring system or the degradation of the pyrrole ring to form the fluorescent products *N*-formylkynurenine or kynurenine [12,13].

Many other oxidative PTMs have been characterized in biological samples, and these have been discussed elsewhere [13]. However, of particular current interest is homocitrullination. The measurement of serum anti-CCP antibodies (antibodies that bind to synthetic cyclic
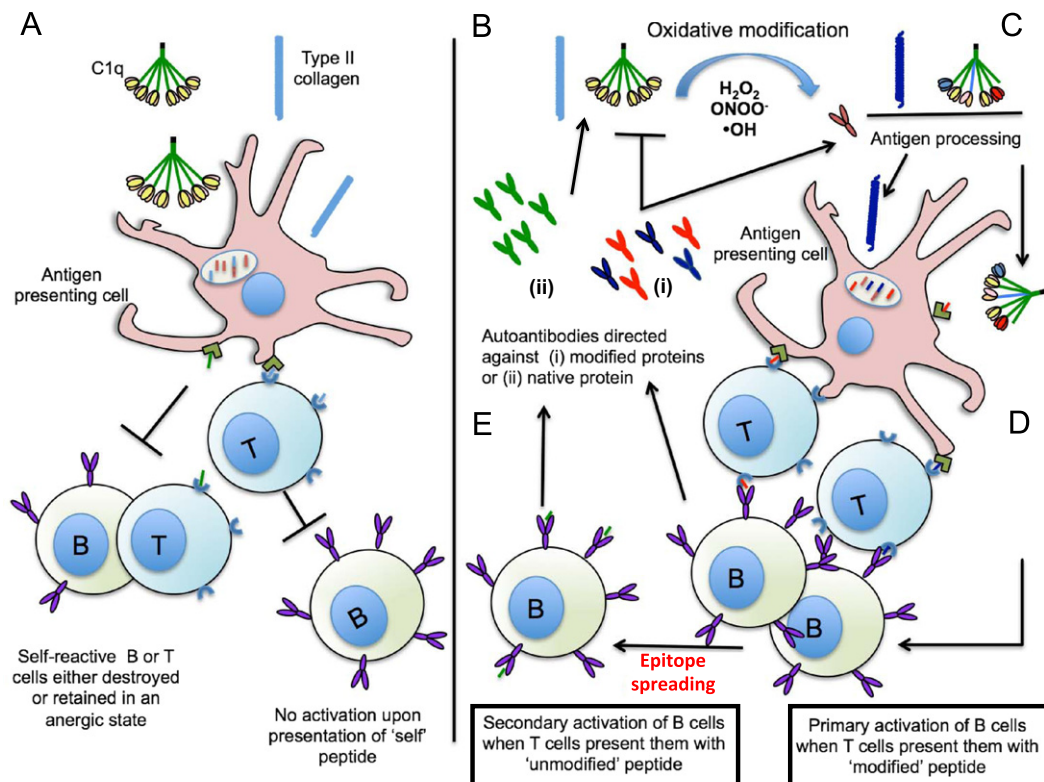
Fig. 1. Oxidative modification of common host proteins such as collagen and complement may lead to breakdown of immune tolerance. (A) Normal lymphocytes do not mount an immune response to self-presented antigens. (B) Free radicals oxidize susceptible amino acids in host molecules at the site of inflammation in genetically susceptible individuals. (C) Modified antigens are taken up by antigen-presenting cells, which can then present the "modified" antigen via MHC class II directly to B cells or to CD4 T cells, which help activate B cells. (D) B cell activation leads to the generation of autoantibodies primarily against modified common host proteins. (E) Upon second or persistent presentation of PTM host antigens, "epitope switching" can occur, resulting in the generation of plasma cells generating autoantibodies against non-PTM host proteins.

citrullinated peptides) is a key diagnostic assay for RA, distinguishing RA from other rheumatic diseases [14–16]. The assay was developed to detect anti-citrullinated protein antibodies (ACPAs). Unfortunately, the antigenic protein(s) responsible for the formation of such antibodies is unknown. Some candidate antigens are fillagrin, vimentin, fibronectin, fibrinogen, and type II collagen (CII). These proteins contain arginine residues, which can undergo a PTM to citrulline, catalyzed by the enzyme peptidyl arginyl deiminase (PAD). The generation of autoantibodies to citrullinated peptides is believed to occur when apoptotic cell death in the joints of patients allows leakage of PAD that posttranslationally converts arginine in the synovium to citrulline [17]. It has therefore generally been assumed that ACPAs arise from enzymatic PTMs within the inflamed RA joint. An alternative possibility, however, is that ACPAs arise from a nonenzymatic PTM of lysine residues. This is an oxidative process that is known to occur in inflammation [18]: neutrophil myeloperoxidase uses H_2O_2 and thiocyanate (SCN^-) as cosubstrates to generate cyanate (OCN^-) and promote protein carbamylation, in which lysine residues are converted to homocitrulline. Because citrulline and homocitrulline are such similar chemical structures, ACPAs may actually be autoantibodies directed against homocitrulline-containing peptide sequences, which cross-react with citrulline-containing sequences. Alternatively, epitope spreading (see above) may explain the presence of ACPAs arising from antigen carbamylation.

Like RA, SLE is an autoimmune inflammatory disease in which oxidative stress appears to be an important pathogenic feature. A further feature of this disease is a deficiency in the clearance of apoptotic cells, and a key pathway of apoptotic cell clearance is the C1q–calreticulin–CD91 pathway. This pathway involves the binding of the complement component, C1q, to the membrane of apoptotic cells. A buildup of apoptotic cells in the blood of SLE patients results in a "reservoir" of posttranslationally modified autoantigens presented by the unremoved apoptotic cells. Donnelly and colleagues [19] have shown that the complement protein, C1q, does not bind to the plasma membranes of apoptotic neutrophils from SLE patients. This suggests a functional deficiency of C1q, a protein that also constitutes an autoantigen in SLE patients.

It has been suggested that this functional deficiency of C1q might be due to the formation of anti-C1q autoantibodies. Why should autoantibodies be generated against C1q, an abundant serum protein that is clearly "self"? Morgan et al. [20] measured the concentrations of modified amino acids in serum proteins from SLE patients by HPLC coupled with UV and fluorescence detection. There were significant increases in serum methionine sulfoxide and 3-nitrotyrosine concentrations in SLE patients compared with healthy control individuals, with the methionine sulfoxide levels correlating with disease activity in SLE patients. Other recent studies [21,22] demonstrated by ELISA that SLE patients had raised serum levels of proteins that had been modified by the lipid peroxidation products malonaldehyde (MDA) and 4-hydroxynonenal (HNE). It was suggested that these chemical modifications constitute neoepitopes that give rise to antigenicity. Antibody titers against MDA- and HNE-modified ovalbumin were elevated in SLE patients and correlated with a clinical index of disease activity (SLEDAI score) [23]. These observations suggest that MDA is systemically present in SLE patients and that MDA modification of antigenic proteins, such as C1q, may play a role in autoantibody formation and disease pathogenesis in SLE. Furthermore, the presence of serum autoantibodies to double-stranded DNA is of diagnostic significance in SLE, and it has been shown that the oxidative modification of DNA generates an antigen with increased avidity of binding to the anti-DNA antibodies in sera from SLE patients compared with sera from healthy individuals [24].

The above discussion indicates the importance of determining plasma autoantibodies directed against oxidatively modified proteins and DNA in autoimmune diseases such as SLE and RA. Such autoantibodies may have diagnostic significance. One of the concerns with all immunoassays for autoantibody production is the degree of sensitivity and specificity. This is compounded by analysis of oxidized antigens. For example, autoantibodies might bind to oxidized antigen with high sensitivity, but such PTMs might lead to the binding of oxidized antigen to other molecules in the serum/plasma. This may, in turn, lead to low sensitivity, because of impedance of the antibody's binding epitope on the oxidized antigen (Fig. 2A). Alternatively, nonspecific binding of serum proteins to the oxidized target protein can elicit false-positive results as these proteins are already in complex with antibodies, leading to decreased specificity (Fig. 2A). The isolation and purification of the antibodies from the complex mixtures of proteins within serum or plasma avoid these potential problems. Such procedures were previously thought to be too cumbersome, but here we provide protocols to isolate purified immunoglobulins from relatively small samples of plasma or serum for conjugation with fluorophores. Such autoantibody–fluorophore conjugates can be used in developing diagnostic assays of autoantibodies against posttranslationally modified antigens in whole cells or isolated components.

Below, we also describe key protocols for producing oxidatively modified proteins and for characterizing the physicochemical properties of the protein autoantigens to which plasma autoantibodies bind. Finally, we describe the quantitation of autoantibody binding by enzyme-linked immunosorbent assays (ELISAs), employing purified proteins that have been oxidatively modified in vitro. The immunoassay described is of the noncompetitive, one-site, type. This has the potential advantage (compared with a two-site immunoassay) that the option is available to modify the protein antigen *after* the immobilization (noncovalent binding) of the protein to a plastic surface, as will be discussed later (see Highlight Box 3).

Collection and processing of extracellular fluids and tissues from patients

Table 1 shows a list of essential clinical information to collect in studies of RA patients and related autoimmune diseases. Similar and analogous clinical information should be considered in other patient groups (e.g., measures of disease activity, disease severity, disease duration, involvement of clinical complications, and comorbidities). In general, the majority of patients with autoimmune disease have a higher incidence of oxidative stress, resulting in greater tissue exposure to ROS than healthy individuals [8,25]. To correlate the generation of oxidatively modified antigens/autoantibody production to disease states, it is critical to record as much clinical information as possible. Blood should be collected into plain tubes (for serum) or heparinized tubes (for plasma) and centrifuged at 2250 g. Freshly aspirated synovial fluid should also be centrifuged under similar conditions, to remove cells and debris. Synovial fluid may be treated with hyaluronidase (30 U/ml for 30 min at 37 °C) to reduce the fluid's viscosity [26], thereby making samples easier to accurately pipette and mix. Plasma, serum, and synovial fluid samples should be divided into aliquots and stored at −80 °C [27].

Isolation of IgG and IgM from small volumes of human serum/plasma, employing fast protein liquid chromatography (FPLC)

Principle

The detection of specific autoantibodies to oxidatively modified proteins, or other oxidatively modified antigens, is usually
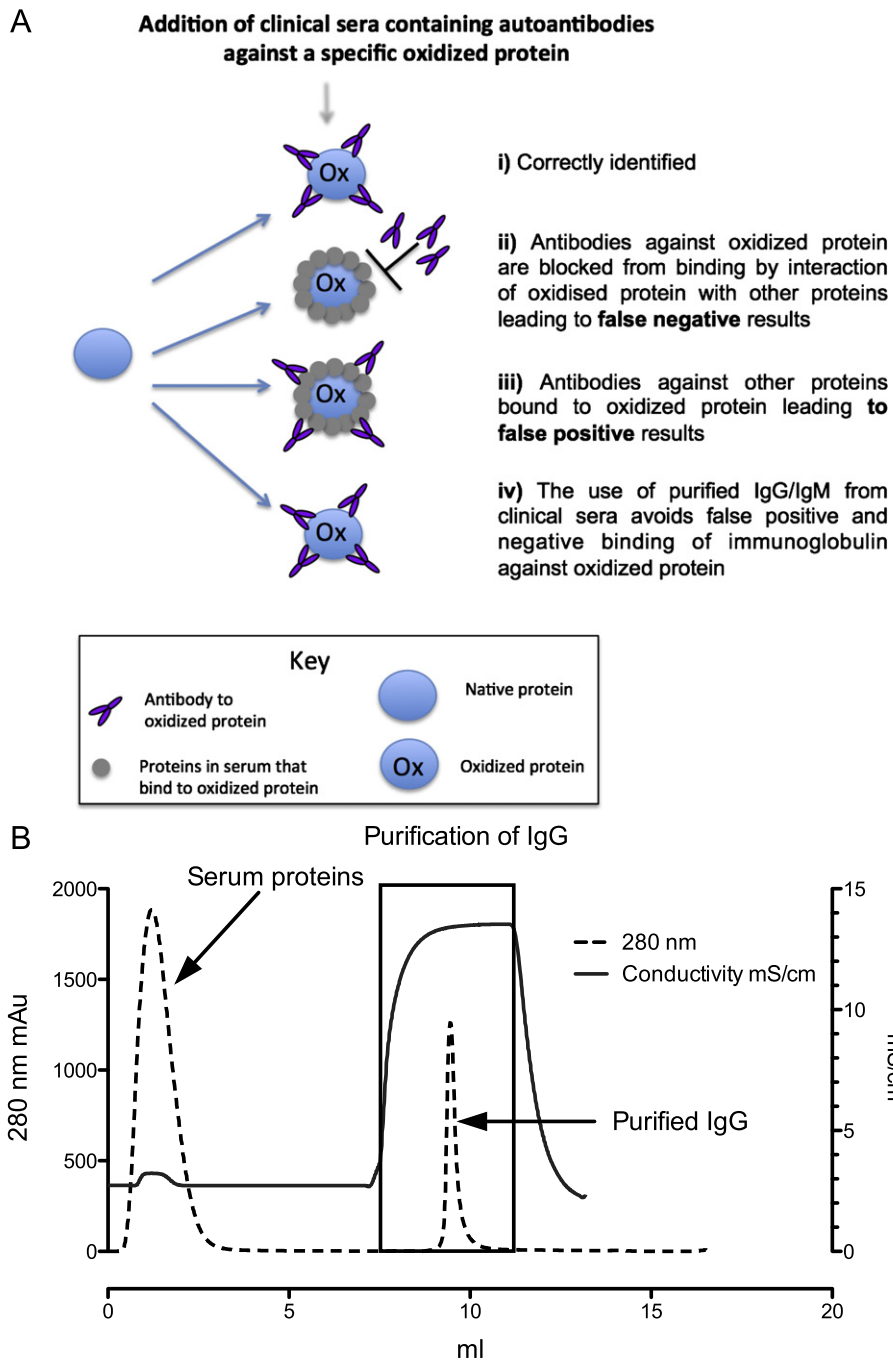
Fig. 2. Isolation of purified immunoglobulin isotypes from small aliquots of human plasma or serum. (A) The rationale for carrying out the purification of immunoglobulin subtypes is driven by the fact that there are different potential explanations (shown here) for an experimental observation that autoantibodies are binding to oxidatively modified proteins when present in complex plasma or serum mixtures. (B) Chromatogram showing the elution profile of IgG from a 0.5-ml aliquot of SLE patient serum. A serum sample was passed down a HiTrap protein G column, and IgG retained on the column was eluted using 200 mM glycine–HCl, pH 2.7. Dotted line, absorbance at 280 nm (A_{280}); solid line, conductivity (salt gradient).

achieved by ELISA or by other immunoassays that provide a protein or other antigen coated onto a well or membrane. As mentioned earlier, the antigenic material that is immobilized may be DNA, lipoprotein, or protein, depending on the purpose of the study. In many published studies, the origin of this antigen (e.g., recombinant protein or protein isolated from human or animal tissue) or the degree of oxidative modification is not cited. Furthermore, antigens can undergo spontaneous oxidation during isolation and purification. Typically, the antigen of interest is then probed with total serum or plasma. This approach has advantages and disadvantages. If the antibody of interest (e.g., against

oxidized low-density lipoprotein) is abundant and specific, then the presence of the antibody can be detected and semiquantified by immunochemistry techniques, e.g., determination of the titer by ELISA. However, if the antibodies against the oxidized antigen are blocked from binding (because of other plasma/serum components binding to the same antigen), then false-negative results will occur. A further drawback is that if the oxidized target antigen binds to other plasma/serum components, the antibody may bind to one or more of the latter proteins, generating a false-positive result (Fig. 2A). These drawbacks can be avoided by initially purifying the antisera from the thousands of other

Table 1
Examples of recommended clinical information to be collected before analyzing extracellular fluids from patients with autoimmune diseases such as rheumatoid arthritis.

Category	Item
Patient information	Patient reference number (to provide anonymity)
	Age
	Gender
	Smoking history
Serology	Full blood count
	Acute-phase protein levels, e.g., C-reactive protein
	Anti-CCP [50]
	Rheumatoid factor (IgG–IgM immune complexes)
Laboratory/clinical tests	Liver function
	Urea and electrolytes
	Urinary creatinine, proteinuria, urinary erythrocytes/field under a light microscope
	Disease activity score (e.g., DAS28 [51], BILAG [52], SLEDAI [53])
	Disease diagnosis (American College of Rheumatology Criteria for RA [54], SLE [55])
Medication	Topical pain relievers
	Oral NSAIDs, e.g., COX-2 inhibitors
	DMARDs, e.g., cytostatic and nucleotide synthesis inhibitors
	Biologic therapeutics, e.g., anti-TNF, anti-CD20, anti-IL-6R

plasma/serum components. This is not a common practice because clinical samples are often obtained in small volumes, ≤ 1.0 ml. However, it is possible to obtain reasonable amounts of purified IgG and IgM isotype antisera from small volumes of serum and plasma.

We have used this approach to facilitate the measurement of anti-C1q antibodies in SLE patients [28]. We found that the ELISA-based measurement of anti-C1q antibodies, either in a purified total IgG fraction or in whole serum, demonstrated higher mean anti-C1q antibody levels in SLE patients compared with healthy individuals. Interestingly, however, the assay utilizing IgG as a source of antibodies provided greater discrimination between patients and controls. Previous work has established that the measurement of anti-C1q antibodies should be carried out in the presence of 1 M NaCl, to inhibit interactions between C1q and confounding factors in serum such as C-reactive protein and rheumatoid factor [29]. These stringent conditions inhibit the interaction of C1q with C-reactive protein, rheumatoid factor, and immune complexes present in the serum. However, when using purified IgG as the source of autoantibodies, the need to use stringent conditions is diminished, as C-reactive protein, rheumatoid factor, and immune complexes are all removed. This argument cannot be made for the potential interaction between C1q and the Fc portion of IgG. Although the affinity of C1q for nonaggregated IgG is low, C1q–Fc interactions will not be abolished when using purified IgG as the antigen source. However, identical amounts of IgG can be added to the assays, and so the level of signal generated from the interaction of C1q with the individual IgG will be constant for all samples. In addition, although the interaction between IgG and its antigen is not eradicated by the addition of 1 M NaCl to samples, the conditions may not be optimal for the interaction, and so genuine antibody–antigen interactions may be inhibited. These considerations exemplify the potential advantages of using purified total immunoglobulins as a matrix in which to detect autoantibodies.

The titers of IgG antibodies versus IgM antibodies can have divergent pathogenic implications. For example, titers of IgM anti-oxLDL antibodies demonstrate a negative correlation with coronary artery disease, although IgG titers demonstrate a positive correlation [30]. IgM anti-oxLDL antibodies arise because an IgM "natural antibody" response can be mounted against oxLDL. Increased levels of oxLDL have been demonstrated in a number of diseases, including SLE and RA [31,32], although the oxidative modification of LDL has been most studied in the context of the development of atherosclerotic plaques. Oxidation of LDL results

in increased immunogenicity of the molecule and ultimately uptake by macrophages and subsequent formation of foam cells, a key constituent of atherosclerotic plaques [33,34]. IgM anti-oxLDL antibodies inhibit the uptake of oxLDL by macrophages and so act in a protective manner [8,35]. Thus, IgM antibodies against oxLDL seem to be protective, whereas IgG antibodies are positively correlated with coronary artery disease.

Materials

- HiTrap Protein G HP and HiTrap Protein IgM HP 1-ml affinity columns (GE Healthcare, Piscataway, NJ, USA; Products 17–0404-03 and 17–5110-01, respectively)
- HiTrap 5-ml desalting column (GE Healthcare; Product 17–1408-01)
- Amicon Ultra-4 centrifugal filter unit with an Ultracel-50 membrane (Millipore, Watford, UK; Product UFC805024)
- Plasma/serum samples (100 μl)
- Binding/elution and equilibration buffers for IgG
  ○ Binding buffer (pH 7.0): 20 mM sodium phosphate buffer (0.327 g $Na_2HPO_4 \cdot 7 H_2O + 0.094$ g NaH_2PO_4, adjusted to pH 7.0 with 1 M HCl and volume brought to 100 ml with dH_2O)
  ○ Elution buffer (pH 2.7): 0.1 M glycine–HCl
  ○ Equilibration buffer (pH 9.0): 1 M Tris–HCl
- Binding/elution and column regeneration buffers for IgM
  ○ Binding buffer (pH 7.5): 20 mM sodium phosphate buffer and 0.8 M $(NH_4)_2SO_4$
  ○ Elution buffer (pH 7.5): 20 mM sodium phosphate buffer
  ○ Regeneration buffer (pH 7.5): 20 mM sodium phosphate buffer with 30% v/v isopropanol

Instrumentation

- AKTA purifier 10 FPLC purification system (GE Healthcare; Product 28–4062-64) and
- UNICORN control software (GE Healthcare; Product 11-0003-38)

Protocol for the purification of IgG from small volumes of serum/plasma

We have developed this protocol to obtain purified IgG or IgM from 100-μl samples of human or animal (e.g., mouse) serum/

plasma, using an AKTA purifier FPLC system with UNICORN software. The system has a programmable autosampler device, thereby facilitating the automated acquisition of purified total IgG from multiple serum samples. The writing of programs to isolate the immunoglobulins is a straightforward procedure and the UNICORN software guides the user through the process step by step. The example below is the method to purify IgG. A similar protocol can be used to purify IgM (described later), but using different binding and elution buffers and a regeneration buffer specific to the IgM protocol.

(1) Prepare a stock solution of 20 mM sodium phosphate binding buffer by combining 0.82 g $Na_2HPO_4 \cdot 7 H_2O$ and 0.24 g NaH_2PO_4 to 245 ml with dH_2O. Use NaOH or HCl to adjust pH to 7.0 and make up finally to 250 ml with dH_2O. Filter the buffer through a 0.45-μm filter and degas the buffer.

(2) Prepare a stock solution of 0.1 M glycine–HCl elution buffer by combining 1.88 g glycine NaH_2PO_4 to 240 ml with dH_2O. Use 5 M HCl to adjust pH to 2.7 and make up finally to 250 ml with dH_2O. Filter the buffer through a 0.45-μm filter and degas the buffer.

(3) Take 100 μl serum/plasma and spin in a benchtop Eppendorf centrifuge at 13,000 rpm for 1 min to remove any precipitates. Add the sample to 400 μl 20 mM sodium phosphate buffer (pH 7.0) and filter through a 0.45-μm filter.

(4) Attach a 1-ml-capacity HiTrap protein G column to the AKTA purifier, using the UNICORN control software, pump out the storage ethanol at 1 ml/min for 5 min. Then, wash the column for 5 min with filtered and degassed dH_2O and equilibrate the column with 5 column volumes (CVs) of binding buffer. Set the pressure alarm default to 0.3 MPa (3 bar) maximum back pressure.

(5) Inject the prepared sample via a 0.5-ml injection loop and run a prewritten program to bind and eventually elute the purified IgG. The unbound sample is washed through the column with 5 CVs binding buffer (Fig. 2B), and bound IgG is subsequently eluted as 0.5-ml aliquots with 5 CVs of elution buffer (Fig. 2B, depicted in boxed area). The pH of each 0.5-ml aliquot of purified IgG is adjusted to pH 7.5 by adding 12.5 μl of equilibration buffer.

(6) The eluted IgG can be relatively dilute, but can be easily concentrated and desalted into the optimum buffer by pooling the IgG fractions and centrifuging them at 3000 g in a 4-ml Amicon ultrafiltration tube with a molecular weight cutoff (MWCO) of 50 kDa, made up to a total volume of 4 ml with the buffer of choice and concentrated to a volume of approximately 100 μl. The IgG can then be topped up with the storage buffer of choice and the protein concentration determined at 280 nm, using the extinction coefficient $E_{280}^{1\%} = 13.6$.

(7) Store the affinity-purified IgG as 50-μl aliquots at $-20\ ^\circ C$. The total IgG fraction, prepared in this manner, is generally stable for years when stored frozen at a concentration of about 1 mg/ml. There is no need to add stabilizers, unless the IgG is going to be stored for a short period at $+4\ ^\circ C$, in which case the use of 0.02% (final concentration) sodium azide is recommended as a preservative.

Protocol for the purification of IgM from small volumes of serum/plasma

A similar procedure can be used to isolate IgM, except for the following modifications:

(1) Prepare a stock solution of 20 mM sodium phosphate/0.8 M ammonium sulfate binding buffer by combining 0.82 g $Na_2HPO_4 \cdot 7 H_2O$ and 0.24 g NaH_2PO_4 and 26.43 g $(NH_4)_2SO_4$, made up to 240 ml with dH_2O. Use NaOH or HCl to adjust the pH to 7.5 and make up finally to 250 ml with dH_2O. Filter the buffer through a 0.45-μm filter and degas the buffer.

(2) Take 100 μl serum/plasma, and remove any precipitate by centrifugation as described above. Add the sample to 400 μl 20 mM sodium phosphate/0.8 M ammonium sulfate binding buffer and filter through a 0.45-μm filter. Note that some methods recommend that solid ammonium sulfate be added to the blood sample, but we find this is unnecessary and can lead to IgM precipitation.

(3) Attach a 1-ml-capacity HiTrap Protein IgM HP to the AKTA purifier and proceed as in step 4 above.

(4) Inject the prepared sample via a 0.5-ml injection loop to bind and eventually elute the purified IgM. If using an AKTA FPLC apparatus, the UNICORN software program provides a "wizard" option that guides the user to write a simple operating procedure, based on the column being used. The unbound sample is washed through the column with 5 CVs of binding buffer (often more washing is recommended, but this normally suffices for such small volumes). The bound IgM is subsequently eluted as 0.5-ml aliquots with 10 CVs of 20 mM sodium phosphate (pH 7.0) elution buffer.

(5) The column is then regenerated by washing it through at 1 ml/min with 7–10 CVs of regeneration buffer.

(6) The IgM can then be desalted, concentrated, and stored as described in steps 6 and 7 of the IgG purification protocol, above.

Non-FPLC isolation of purified IgG and IgM

If an FPLC system is not available, or prohibitively expensive to acquire, then a more labor-intensive technique employing the same HiTrap columns and buffers as above can be performed. Simply wash the columns and equilibrate as above and elute the unbound serum protein in 0.5-ml aliquots. Once the columns are washed, use fresh Eppendorf tubes to elute the purified IgG or IgM as 0.5-ml fractions manually by passing the elution buffer contained in a 10-ml syringe through the column gently. Identify which Eppendorf tubes contain the eluted immunoglobulins by placing each sample in a quartz cuvette and performing a spectrophotometric analysis at 280 nm. The resulting aliquots of purified IgG/IgM can be desalted/concentrated and stored as described above.

Fluorescence labeling of submilligram amounts of immunoglobulins and other proteins

Principle

Fluorescein isothiocyanate (FITC)-based labeling techniques have been used for many years to fluorescently label proteins. Fluorescein conjugation to immunoglobulins from patient samples allows monitoring of direct autoantibody binding to cell surface and intracellular proteins by immunochemistry, immunoblotting, and flow cytometry. Fluorescein-conjugated proteins are very stable and sensitive to detection with an absorption maximum of 495 nm and emission maximum at 525 nm. FITC reacts with free amino groups in amino acids, and the positively charged amino acid lysine is readily labeled by FITC. This must be taken into consideration if a specific PTM is being investigated that involves the same amino groups (e.g., lysine methylation or carbamylation).

In cases in which antibody–antigen binding might be affected by the derivatization of key amino groups in an antibody or

protein antigen, for example, the side-chain amino groups of crucial lysine residues, consideration should be given to carrying out experiments using a fluorescence labeling strategy that avoids the derivatization of amino groups. For example, fluorophore conjugation can be carried out using either sulfhydryl-reactive maleimide-activated fluorophores such as fluorescein–5-maleimide [36] or fluorophore hydrazides, which react covalently with the carbohydrate moieties located within the Fc portion of antibody molecules or within glycoprotein antigens [37].

An advantage of labeling immunoglobulin or other proteins with FITC is that it is a relatively simple and inexpensive technique to perform. There are a number of commercial kits on the market that provide all the items required to perform FITC conjugation of protein ranging from 1 to 5 mg. In many cases, the amounts of proteins available to label for research purposes are often < 1 mg. In this method, we describe a procedure for labeling submilligram quantities of antibodies and other proteins with FITC for use in the direct detection of autoantigens in cells and isolated proteins.

Materials

- Conjugation buffer: 0.1 M sodium carbonate–bicarbonate (pH 9.0)—prepared from anhydrous sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$)
- Amicon Ultra-4 centrifugal filter unit with Ultracel-50 membrane (Millipore; Product UFC805024)
- FITC (MW 389) (Sigma, St. Louis, MO, USA; Product F4274)
- HiTrap 5-ml desalting column (GE Healthcare; Product 17–1408-01)

Instrumentation

- AKTA purifier 10 FPLC purification system (GE Healthcare; Product 28–4062-64) and
- UNICORN control software (GE Healthcare; Product 11-0003-38)

Protocol for the FITC conjugation of submilligram amounts of immunoglobulins and other proteins

(1) Prepare a stock solution of 0.1 M sodium carbonate conjugation buffer by adding 1.06 g Na_2CO_3 to 100 ml dH_2O and separately 0.84 g $NaHCO_3$ to 100 ml dH_2O. On a magnetic stirrer, add the Na_2CO_3 to the $NaHCO_3$ until pH 9.0 is achieved.

(2) Take a protein sample at a concentration $\geq$ 0.2 mg/ml and place 1 ml in a 4-ml Amicon ultrafiltration tube (MWCO 50 kDa—if protein of interest has a MW of > 50 kDa, e.g., IgG has a MW of 150 kDa and C1q a MW of 460 kDa) topped up to 4 ml with the 0.1 M conjugation buffer; centrifuge at 3000 g until the protein is retained in a 200-µl volume (to achieve a 1 mg/ml concentration).

(3) Prepare a 0.5 mg/ml (0.128 mM) FITC solution in conjugation buffer, by adding 5 mg FITC to 10 ml of conjugation buffer. Place the protein to be FITC conjugated in a 1-ml-capacity glass beaker containing a magnetic stir bar. Slowly add 50 µl of the FITC solution. Cover the beaker in aluminum foil to protect from light and stir for 2 h at room temperature.

(4) Attach a 5-ml-capacity desalting column packed with Sephadex G-25 superfine to the AKTA purifier. Using the UNICORN control software, pump out the storage ethanol at 1 ml/min for 10 min. Wash the column for 10 min with filtered and degassed dH_2O and equilibrate the column with 5 CVs of desired buffer (commonly phosphate-buffered saline, pH 7.0).

Set the pressure alarm default to 0.3 MPa (3 bar) maximum back pressure.

(5) Make up FITC-conjugated sample to 0.5 ml with the desired buffer. Inject the sample via a 0.5-ml injection loop and run a prewritten program set to give a flow rate of 1 ml/min for the desired filtered, degassed buffer. Set the program to collect 0.5-ml fractions, with dual absorbance detection set at 280 nm (protein absorbance maximum) and 495 nm (FITC absorbance maximum). The FITC-conjugated immunoglobulin or protein elutes from the column first in the desired buffer and presents with a 280/495 nm absorbance spectrum. This confirms that the protein is indeed FITC conjugated (Fig. 3A). Several minutes later, the free FITC is eluted through the column as confirmed by its 495-nm absorbance spectrum (Fig. 3A).

(6) The eluted FITC-conjugated protein may be relatively dilute, but can be concentrated by centrifugation at 3000g in a 4-ml Amicon ultrafiltration tube (MWCO 50 kDa) to the desired volume and concentration. Filter sterilize before use with cells.

(7) It is important not to overlabel the protein with FITC as this may lead to false-positive and high fluorescence-background results, when applied to immunohistochemistry and flow cytometry analysis. It is generally accepted that FITC-conjugated proteins should have a molar ratio of fluorescein/protein (F/P) between 2.0 and 6.0, to confirm adequate labeling.

(8) To determine the F/P molar ratio, a constant value (C) for the FITC conjugation of your protein of choice must be calculated.
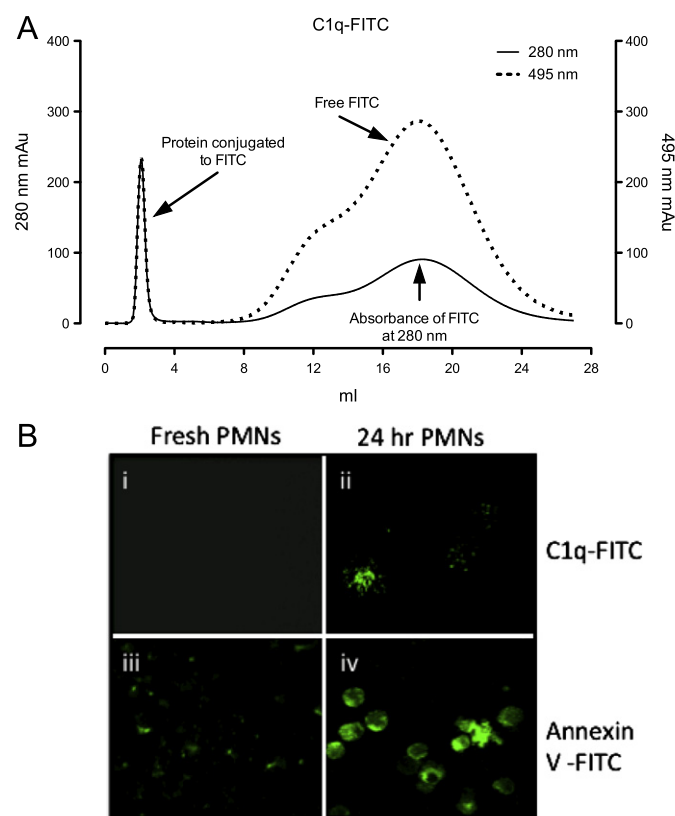

Fig. 3. FITC labeling of C1q and direct binding to apoptotic cell surfaces. (A) FPLC chromatogram of FITC-conjugated antibody and unbound FITC. A polyclonal rabbit anti-C1q antibody was incubated with FITC in sodium carbonate buffer. The FITC-conjugated antibody was separated from unbound FITC by FPLC, using a 5-ml desalting column. The absorbance of the eluent was measured at 495 and 280 nm to monitor FITC and protein elution and to calculate the F/P ratio of the conjugated antibody, which was determined to be 4.49. (B) Immunohistochemistry of C1q–FITC binding to freshly isolated and 24-h cultured neutrophils (top). The bottom row shows the same cells binding to the apoptotic cell marker annexin V–FITC.

Where MW is the molecular weight of the protein, 389 is the MW of FITC, 195 is the absorbance of a 0.1% (w/v) solution ($E^{0.1\%}$) of bound FITC at 490 nm:

$$C = \left(MW \times E_{280}^{0.1\%}\right)/(389 \times 195).$$

The $E_{280}^{0.1\%}$ values for commonly conjugated immunoglobulins are for IgG, 1.36; for IgM, 1.18; and for F(ab')^2, 0.15. In the non-immunoglobulin protein example provided here—the $E_{280}^{0.1\%}$ for the first component of complement, C1q (MW 460,000) is 0.68. Therefore the C value is calculated as follows:

$$C = (460,000 \times 0.68)/(389 \times 195) = 4.12.$$

In the example given in Fig. 3A, the experimental absorbances can be inserted into the general formula below to calculate the molar F/P ratio, where $0.35 \times A_{495}$ is the correction factor due to the absorbance of FITC at 280 nm (the effect of which can be seen in Fig. 3A):

$$Molar\frac{F}{P} = \frac{MW}{389}$$
$$\times \frac{A_{495}/195}{A_{280}-(0.35 \times A_{495})/E^{0.1\%}} = \frac{A_{495} \times C}{A_{280}-(0.35 \times A_{495})}.$$

In the example given, where C1q has been labeled,

$$Molar\frac{F}{P} = \frac{460,000}{389}$$
$$\times \frac{0.23/195}{0.23-(0.35 \times 0.23)/0.68} = \frac{0.23 \times 4.12}{0.23-(0.35 \times 0.23)}.$$

The molar F/P ratio is $0.95/0.15 = 6.3$. This would normally be considered slightly overlabeled, an F/P molar ratio of 2.0–6.0 being optimal. The experiment could be repeated by simply adding 50 μl of a 1:2 dilution of the originally prepared FITC solution to another preparation of protein. Steps 3–8 of the process are then repeated. It is common to have to conduct some trial runs to optimize FITC labeling, as the efficiency of FITC conjugation varies for each protein and is dependent upon the number of exposed lysine residues available for labeling.

(9) The FITC-conjugated protein can then be used to study direct binding to cells before and after various treatments. In the example given in Fig. 3B, the ability of C1q to bind directly to fresh or 24-h-old, aged, neutrophils is shown. Using the C1q–FITC-conjugated protein, there is, of course, no need to detect the binding of C1q using primary and secondary antibodies.

Oxidative and nitrative modifications of protein antigens

Principle

Because autoantibodies often recognize proteins that have undergone PTMs in vivo, it is necessary to generate the same modifications in isolated proteins in vitro to detect these auto-antibodies by immunochemical methods. The protocols given below, for the generation of PTMs, represent only a fraction of the diverse range of potential modifications.

Protocol for the generation of PTMs by exposure of a target protein to H_2O_2, $H_2O_2+Cu^{2+}$ ions (thereby generating $^{\bullet}OH$), HOCl, or $ONOO^-$

(1) Before modification, the target protein (typically 1 mg/ml) should be diluted (1:1) in 0.4 M sodium phosphate buffer, pH 7.4, or other chosen buffer (see Box 1, Buffer considerations).

Box 1

Buffer considerations

(1) The reactions of ROS/RNS and their subsequent reactions with proteins are often dependent on the pH of the solution. Be wary of adding a ROS/RNS-generating system to a nonbuffered, or insufficiently buffered, solution. This may result in reactions taking place at a nonphysiological pH, thereby altering the chemical modifications that are observed. It is particularly important to control the change in pH that may otherwise be caused by the addition of oxidants such as HOCl, pK_a 7.54, and $ONOO^-$, pK_a 6.8. The protocol provided in Box 2 will result in the synthesized $ONOO^-$ being present in a relatively high concentration of NaOH, which will maintain the stability of $ONOO^-$ in stock solution. When considering appropriate dilutions of the $ONOO^-$ solution, prior to addition of $ONOO^-$ to buffered target proteins, the potential pH-shifting effects of the NaOH should be remembered.

(2) Tris–HCl (as well as Good's buffers) may sometimes be a useful buffer for carrying out some oxidative modifications. However, given the $^{\bullet}OH$-scavenging capacity of Tris, a sodium phosphate buffer may be preferable. On the other hand, when studying transition-metal-catalyzed radical reactions, it should be remembered that iron ions readily form insoluble ferric hydroxyl phosphate complexes. Thus no single buffer is suitable for the generation of all types of oxidative protein modifications. In this context, consideration should be given to the desired pH, the presence/absence of transition metal ions, and the potential reactions of the buffer components with the specific type of ROS/RNS being produced in the system.

(3) Beware of the potential for unanticipated Fenton chemistry within the protein modification system. Laboratory water and reagents are typically contaminated with micromolar concentrations of transition metal ions, such as iron or copper, which can catalyze $^{\bullet}OH$ production from H_2O_2, through the Fenton reaction. Given that the oxidizing species H_2O_2 and $^{\bullet}OH$ are known to exhibit different reactivities and modify different amino acid residues, treatment of all reagents and buffers with Chelex-100 resin (removes transition metal ions [18,42]) facilitates the use of chemical modification systems that are free of Fenton-derived $^{\bullet}OH$ formation. For Chelex-100 treatment of solutions, the resin can be prepared in a column or mixed with solutions and then pelleted by centrifugation before pipetting off the treated solution (Bio-Rad Analytical Grade Chelex resin, 100–200 mesh, sodium form, Cat. No. 142-2832).

(4) Working with some ROS/RNS, for example H_2O_2 and $ONOO^-$, presents challenges due to their potential rapid decomposition in neutral, aqueous solutions (in the case of H_2O_2, the possible Fenton reaction). It is therefore essential to prepare buffered protein solutions before adding ROS/RNS and to limit the number of dilutions of the stock oxidant.

---

(2) Oxidants are prediluted from stock solutions immediately before incubation with the protein. Solutions of H_2O_2, HOCl, or $CuCl_2$ are diluted in pH-adjusted dH_2O, whereas $ONOO^-$

solutions are diluted in 1 mM NaOH (see Box 2, Peroxynitrite synthesis and Refs. [38,39]). H_2O_2 may be added to the target protein solution, either alone or immediately followed by the addition of $CuCl_2$ to generate $^\bullet OH$. Oxidants are mixed with buffered solutions of protein by vortex mixing for 5 s and reactions are allowed to proceed for 1 h at room temperature in the dark.

(3) For the purposes of generating PTMs, proteins are typically exposed to final concentrations of 1–100 mM H_2O_2 (±0.1 mM $CuCl_2$), 0.01–10 mM HOCl, or 0.01–10 mM $ONOO^-$. Clearly this is not a physiological range of concentrations of oxidant, nor is it intended to be so. For the purposes of this protocol, the intention is to generate an optimal amount of oxidatively modified protein to which autoantibodies that recognize the modified protein will optimally bind. It is necessary to empirically determine the optimal concentration of each oxidant, such that chemical modifications in the selected protein are induced without completely destroying the primary structure of the protein (e.g., no visible bands remaining on a Coomassie-stained SDS–PAGE gel).

Generation of PTMs by the carbamylation of a target protein

The target protein is carbamylated by incubation with 100 μM potassium cyanate (KOCN; Axxora, UK) in 50 mM sodium phosphate buffer, pH 7.0, at 37 °C for 8 h, as described by Wang et al. [18].

Generation of PTMs by exposure of a target protein to lipid peroxidation products

The target protein is modified with 100 mM MDA as described by Kim et al. [40]. First, MDA is generated by the acid hydrolysis (0.2 ml of 12 M HCl) of 0.165 ml of malonaldehyde bis(dimethylacetal) (Axxora) at room temperature. After this, 4.8 ml of 100 mM sodium phosphate buffer (pH 6.4) is added, and the solution is adjusted to pH 6.4 with 10 M NaOH. The modification of the target protein is initiated by mixing equal volumes of protein solution, in 10 mM sodium phosphate buffer containing 0.01% EDTA (pH 7.4), with 100 mM freshly prepared MDA in 100 mM sodium phosphate buffer (pH 6.4). The mixture is incubated at 37 °C for 24 h. The reaction is stopped by dialysis against 50 mM sodium phosphate buffer containing 0.01% EDTA (pH 7.4) for 24 h at 4 °C. Samples containing PTMs that result from exposure to lipid peroxidation products should be analyzed and applied in autoantibody measurements (see below), using *freshly prepared material*. This is important because reactions may continue to occur within the sample, whereas freezing some PTM proteins can result in precipitation. It should also be noted that posttranslationally modified proteins will of course contain a range of PTMs, potentially representing variable relevance to the situation *in vivo*. These factors highlight the importance of carrying out a structural characterization of the generated PTMs, which will be described later.

Generation of PTMs by treatment of a protein with glucose oxidation products

To induce extensive PTMs, the target protein (about 0.5–5 mg/ml, depending on the molecular weight of the protein) is treated with 30 mM methylglyoxal diluted in 50 mM sodium phosphate buffer, pH 7.4, for 24 h at 37 °C [41]. After incubation, the reaction mixture is washed extensively using a centrifugal ultrafiltration device (e.g., Amicon Microcon filter from Millipore; the MWCO of the filter should be tailored to the size of the protein antigen under investigation). The reacted mixture is pipetted into the top of the device and centrifuged (13,000 rpm, 1 h, 4 °C) to remove any unreacted methylglyoxal. The mixture is then washed with water (4 °C) and centrifuged under the same conditions. This procedure should be repeated four times to ensure the removal of methylglyoxal.

Analysis of chemically modified proteins

Principle

Once a protein has been subjected to PTMs in vitro, it is important to confirm the chemical nature and extent of the modifications and whether these reflect the modifications that occur in vivo. This can be achieved by the complementary techniques of SDS–PAGE, FPLC, and mass spectrometry, as described below. Three-dimensional scanning fluorescence spectra may also be obtained [6], in which simultaneous excitation (e.g., 250–450 nm) and emission (e.g., 250–600 nm) spectra are recorded. Of course, other analytical methods can also be informative.

SDS–PAGE

Native or modified protein (5 µg) in sample buffer is subjected to reducing/denaturing SDS–PAGE [47]. The gel is stained with Coomassie brilliant blue and, after destaining, image capture and analysis are carried out digitally. As an example, the results obtained from reducing SDS–PAGE analysis of native and oxidatively modified CII are shown in Fig. 4A. It is important to note that oxidative modifications can induce the formation of high-molecular-weight species, and so the removal of the stacking gel, for either SDS–PAGE or immunoblotting, is not recommended.

Analysis by fast protein liquid chromatography

Modified protein (50–200 µg in a final volume of 100 µl) is loaded onto an AKTA FPLC system (GE Healthcare) and passed down a size-exclusion column, selected on the basis of the molecular weight of the protein under study (for example, Superdex 75 column, MW range 3–70 kDa, Product 17–5174-01, or Superdex 200 column, MW range 10–600 kDa, Product 17–5175-01; GE Healthcare) at a flow rate of 1.0 ml min^{-1}. Before injection, the column is equilibrated with 1 CV of PBS, pH 7.4. Proteins are eluted from the column using PBS, and the absorbance of the eluent is measured at 215 nm (peptide bond absorbance) and 280 nm (aromatic amino acid absorbance). The formation of fluorescent products such as *N*-formylkynurenine is monitored by inline fluorescence detection (for example, Hitachi F-1050 fluorescence detector) with excitation at 360 nm and emission at 454 nm. If it is suspected that the PTM has generated protein scission products (e.g., based on information from SDS–PAGE analysis), it is possible to analyze the released peptides using a high-resolution peptide chromatography column, such as the Superdex Peptide 10/300 GL column (GE Healthcare; Product 17–5176-01).

Mass spectrometry

Covalently modified proteins may be analyzed by mass spectrometry to characterize the precise chemical nature of the induced modifications. The use of this technique in analyzing oxidative/nitrative modifications has been extensively reviewed elsewhere [48,49]. Typically, liquid chromatography–electrospray ionization–tandem mass spectrometry is used to sequence the modified protein, taking into account that specific chemical modifications of target amino acids will cause characteristic *m/z* (mass/charge) shifts in the secondary ions. MALDI/–ToF–MS
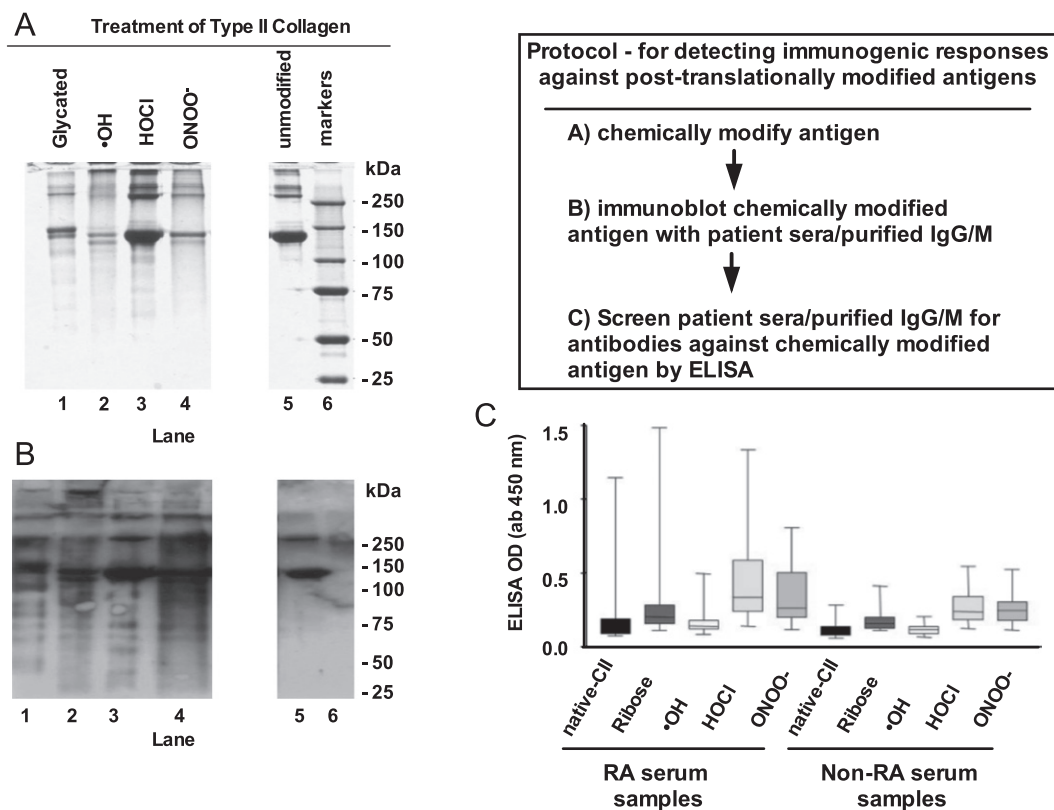

Fig. 4. Analysis of the effects of various posttranslational modifications on the ultrastructure and antigenicity of an autoantigen—in this case type II collagen. (A) Type II collagen was treated with ROS/RNS and analyzed by SDS–PAGE. Note that the stacking gel has not been removed, as this allows the observation of high-molecular-weight aggregates, if present, which have not entered the separation gel. Lane 1, glycation by ribose caused a shift in the CII α-chain band to a position of slightly higher molecular weight, as well as fragmentation. Lane 2, hydroxyl radical (•OH) caused a loss of the intact CII polypeptide, together with both fragmentation and formation of high-molecular-weight aggregates that did not migrate from the sample loading slot. Lane 3, hypochlorous acid (HOCl) treatment resulted in both aggregation and fragmentation. Lane 4, peroxynitrite (ONOO$^-$) treatment resulted in a loss of intact CII polypeptide, together with fragmentation. Lane 5, the α-chain polypeptide of native untreated CII. The positions of the molecular weight markers (in kDa) are shown on the right. Modified from Nissim et al. [6], with permission. (B) Qualitative analysis of autoantibodies present in human serum and directed against various oxidatively modified forms of type II collagen. To obtain the immunoblot shown, the proteins that had been separated by SDS–PAGE were blotted from the gel onto a nitrocellulose membrane and probed with serum from a patient with RA. This was followed by detection with IgG–horseradish peroxidase reagents. Modified from Nissim et al. [6], with permission. (C) Quantitative analysis of the binding of serum autoantibodies, as assessed by ELISA detection of antibodies to modified and native CII in serum samples from patients with RA ($n=21$) and patients with other inflammatory joint diseases (non-RA) ($n=26$). A significant increase in the binding to modified CII was detected in all RA sera compared with binding to native CII (*$P=0.0023$), except for CII modified by hydroxyl radical (**$P=0.19$ versus binding to native CII). Box plots show the 25th and 75th percentiles, and the horizontal lines show the median (50th percentile). Bars outside the boxes indicate the minimum and maximum values. Modified from Nissim et al. [6], with permission.

(matrix-assisted laser desorption/ionization–time of flight–mass spectrometry) of trypsin digests of the modified protein may also be useful as a relatively quick screen to identify modifications within the released peptides, particularly when the likely nature of the modification(s) is already known. Finally, linear MALDI–ToF–MS may be useful if the target protein is of a relatively low molecular weight.

Detection of autoantibodies against oxidatively modified proteins by immunoblotting

Principle

Immunoblotting of native proteins, and proteins that have been subjected to PTMs, can be carried out by detecting the binding of autoantibodies within whole serum samples or isolated IgG (isolated from serum as described above). Fig. 4B shows an example of such an immunoblot, which employed native and modified CII as the target antigen and assessed the binding of IgG in an RA serum sample.

Protocol

(1) Modified and native proteins (2 µg of each) are subjected to reducing SDS–PAGE (7.5% cross-linked gel) and electroblotted onto a nitrocellulose/PVDF membrane (BDH Chemicals).
(2) After being blocked with 2% dried milk powder in PBST (PBS containing 0.1% (v/v) Tween 20), each nitrocellulose membrane is incubated with a 1:200 dilution of a serum sample for 2 h at room temperature.
(3) Each membrane is then incubated with anti-human IgG–horseradish peroxidase (HRP; Sigma) for 2 h at 37 °C.
(4) Finally, each membrane is washed extensively (at least four times) with PBST before being developed by enhanced chemiluminescence, using for example, a Chemidoc XRS system with Quantity One software (Bio-Rad Laboratories, Hemel Hempstead, UK).

Measurement by ELISA of autoantibodies against oxidatively modified proteins

Principle

The method below has been applied successfully to detect, in RA serum samples, autoantibodies against various PTMs of CII (Fig. 4C and [6]). This protocol may be applied (either as is or in modified form—see, for example, Box 3, Issues relating to the immobilization and retention of chemically modified antigens) to the quantitation of autoantibodies that recognize other posttranslationally modified proteins.

Protocol

(1) Unmodified CII and modified forms of CII are prepared (see above). Samples (10 µg protein) are diluted in sodium carbonate buffer, pH 9.6 (0.015 M Na_2CO_3/0.035 M $NaHCO_3$), to a final volume of 100 µl, and coated onto 96-well Maxisorp plates (Nunc) overnight at 4 °C.
(2) Plates are then washed three times with PBS.
(3) Next, each well is treated with blocking buffer (2% (w/v) dried milk powder in PBS) for 2 h at 37 °C.
(4) Plates are washed (three times with PBS) and then, to individual wells, 1:200 diluted serum (diluted in blocking buffer), final volume 100 µl, is added. The plates are then incubated for 2 h at 37 °C.

Box 3

Issues relating to the immobilization and retention of chemically modified antigens on protein absorption plates used for ELISAs

Careful consideration should be given as to the stage of the ELISA at which the PTM of a protein antigen is carried out

This may well involve pilot experiments to determine the optimal strategy for immobilization and retention of the target (antigenic) protein. An obvious approach is to carry out the PTMs to the target protein while the protein is in bulk solution, after which the protein is immobilized on the surface of a 96-well plate. Clearly, however, bulk-phase PTMs may result in the modified protein absorbing to the plate to a different extent, compared to the native protein. This may not be an issue if the immobilized protein is always in excess relative to the antibody that is recognizing it. An alternative strategy is to immobilize the native target protein on the surface of a 96-well plate and afterward to carry out PTM reactions. Solid-phase (plate-bound) proteins may be modified by the addition of 50 µl of oxidant solution to each well, which already contains 50 µl of 0.4 M sodium phosphate buffer, pH 7.4. Incubation is then carried out for 1 h at room temperature. Possible factors contributing to the loss of protein from the solid-phase include the following

- Oxidants may cause the cleavage of the polypeptide backbone of a protein or disrupt the protein's noncovalent interactions with the ELISA plate, subsequently resulting in the loss of protein and/or peptides from the plate.
- The sufficiency of the buffer in which the protein is modified may be inadequate. Be aware that any pH changes during the PTM reaction may affect the retention of the target protein on the ELISA plate surface as well as altering the reactions of oxidants with the constituent amino acids of the protein (this is particularly pertinent for HOCl and $ONOO^-$ modifications).

A method for confirmation of protein retention on protein absorption plates—Coomassie retention assay

- Protein (0.2–5 µg) is coated onto a 96-well ELISA plate in sodium carbonate buffer, pH 9.6, overnight at 4 °C.
- The plate is washed twice in PBST and twice in PBS, and then PTM of the target protein is carried out, as described above.
- The plate is washed again and then incubated with 50 µl of Coomassie brilliant blue stain for 1 h. A 100-ml Coomassie blue solution comprises 120 mg of Coomassie G-250 dissolved in methanol (45 ml), 1 M acetic acid (10 ml), and dH_2O (45 ml).
- The plate is washed six times with dH_2O, and then 100 µl of Coomassie destain solution is added to each well. A 100-ml destain solution comprises methanol (45 ml), 1 M acetic acid (10 ml), and dH_2O (45 ml). The plate is then incubated at room temperature for 2 h with agitation.
- The absorbance of the destain solution is read at 595 nm in a spectrophotometric plate reader.

The utility of competitive ELISAs

Consideration should be given to the fact that when an antigen is immobilized on plastic, there may be steric limitations placed on the interaction of the antigen with the primary antibody. This

issue can be addressed by examining the competitive binding of the antigen after it has been subjected to PTM [31]. In a competitive ELISA, the posttranslationally modified antigen will be present in free solution and should thereby be able to interact with the antibody in the same manner as when present in vivo.

(5) Plates are washed three times with PBST and then incubated with 100 µl of a 1:1000 dilution of anti-human IgG–HRP (Sigma) in blocking buffer for 2 h at 37 °C.

(6) Plates are washed (three times with PBST), and then 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma; 100 µg/ml in 100 mM sodium acetate, pH 6.0) is added and left for up to 30 min in the dark.

(7) Subsequently, the reaction is stopped by the addition of 50 µl of 1.0 M H_2SO_4. The absorbance is read at 450 nm using a spectrophotometric plate reader (e.g., GENios plate reader; Tecan, Reading, UK).

(8) N.B. All samples should be analyzed in triplicate. Often data are initially expressed as optical density (OD) at the selected absorbance wavelength, in this case 450 nm (see Fig. 4C).

(9) Data can be normalized by calculating the ELISA unit (EU) value of a sample, which is defined as its $OD_{450\,nm}$ expressed as a proportion of the mean $OD_{450\,nm}$ of a reference sample (one patient serum sample with high autoantibody levels against the protein antigen of interest): $EU = mean\ OD_{450\,nm}$ (sample)/mean $OD_{450\,nm}$ (reference) × 100.

Conclusion

Here we have described a set of interrelated protocols for the determination of serum autoantibodies against characterized, oxidatively modified, proteins in clinical samples. In addition to serum-based analyses, this same methodological approach is also applicable to autoantibody detection and characterization in a variety of human and rodent extracellular fluids and tissues, including plasma, knee-joint synovial fluid, other inflammatory exudates, tissue extracts, etc. The identification of clinically significant autoantibodies that recognize specific PTMs has already generated important diagnostic tests, which have been adopted in clinical practice. An example is the anti-CCP test used for the diagnosis of RA. Such advances have also provided key mechanistic insights into the pathogenesis of autoimmune inflammatory diseases. We propose that the application of the types of protocols described here will uncover novel autoantibodies against oxidatively modified proteins in autoimmune disease patients and that some of these autoantibodies will be diagnostically significant.

Acknowledgments

References

[1] Blake, D. R.; Bodmyali, T.; Stevens, C. R.; Winyard, P. G. Introduction. In: Winyard, P. G., Blake, D. R., Evans, C. H., editors. *Free Radicals and Inflammation.* Basel: Birkhäuser; 2000. p. 1.

[2] Eggleton, P.; Haigh, R.; Winyard, P. G. Consequence of neo-antigenicity of the 'altered self'. *Rheumatology (Oxford)* **47:**567–571; 2008.

[3] Fang, J. C.; Kinlay, S.; Behrendt, D.; Hikita, H.; Witztum, J. L.; Selwyn, A. P.; Ganz, P. Circulating autoantibodies to oxidized LDL correlate with impaired coronary endothelial function after cardiac transplantation. *Arterioscler. Thromb. Vasc. Biol.* **22:**2044–2048; 2002.

[4] Peters, M. J.; van Halm, V. P.; Nurmohamed, M. T.; Damoiseaux, J.; Tervaert, J. W.; Twisk, J. W.; Dijkmans, B. A.; Voskuyl, A. E. Relations between autoantibodies against oxidized low-density lipoprotein, inflammation, sub-clinical atherosclerosis, and cardiovascular disease in rheumatoid arthritis. *J. Rheumatol.* **35:**1495–1499; 2008.

[5] Kirkham, P. A.; Caramori, G.; Casolari, P.; Papi, A.; Edwards, M.; Shamji, B.; Triantaphyllopoulos, K.; Hussain, F.; Pinart, M.; Khan, Y.; Heinemann, L.; Stevens, L.; Yeadon, M.; Barnes, P. J.; Chung, K. F.; Adcock, I. M. Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of COPD. *Am. J. Respir. Crit. Care Med.* **184:**796–802; 2011.

[6] Nissim, A.; Winyard, P. G.; Corrigall, V.; Fatah, R.; Perrett, D.; Panayi, G.; Chernajovsky, Y. Generation of neoantigenic epitopes after posttranslational modification of type II collagen by factors present within the inflamed joint. *Arthritis Rheum.* **52:**3829–3838; 2005.

[7] Eggleton, P.; Reid, K. B.; Tenner, A. J. C1q—how many functions? How many receptors? *Trends Cell Biol.* **8:**428–431; 1998.

[8] Phillips, D. C.; Dias, H. K.; Kitas, G. D.; Griffiths, H. R. Aberrant reactive oxygen and nitrogen species generation in rheumatoid arthritis (RA): causes and consequences for immune function, cell survival, and therapeutic intervention. *Antioxid. Redox Signaling* **12:**743–785; 2010.

[9] Fathman, C. G.; Soares, L.; Chan, S. M.; Utz, P. J. An array of possibilities for the study of autoimmunity. *Nature* **435:**605–611; 2005.

[10] Doyle, H. A.; Mamula, M. J. Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol.* **22:**443–449; 2001.

[11] Trinder, P. K.; Maeurer, M. J.; Stoerkel, S. S.; Loos, M. Altered (oxidized) C1q induces a rheumatoid arthritis-like destructive and chronic inflammation in joint structures in arthritis-susceptible rats. *Clin. Immunol. Immunopathol.* **82:**149–156; 1997.

[12] Stadtman, E. R.; Levine, R. L. Protein oxidation. *Ann. N. Y. Acad. Sci.* **899:**191–208; 2000.

[13] Winyard, P. G.; Ryan, B.; Eggleton, P.; Nissim, A.; Taylor, E.; Lo Faro, M. L.; Burkholz, T.; Szabo-Taylor, K. E.; Fox, B.; Viner, N.; Haigh, R. C.; Benjamin, N.; Jones, A. M.; Whiteman, M. Measurement and meaning of markers of reactive species of oxygen, nitrogen and sulfur in healthy human subjects and patients with inflammatory joint disease. *Biochem. Soc. Trans.* **39:**1226–1232; 2011.

[14] Mydel, P.; Wang, Z.; Brisslert, M.; Hellvard, A.; Dahlberg, L. E.; Hazen, S. L.; Bokarewa, M. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. *J. Immunol.* **184:**6882–6890; 2010.

[15] Shi, J.; Knevel, R.; Suwannalai, P.; van der Linden, M. P.; Janssen, G. M.; van Veelen, P. A.; Levarht, N. E.; van der Helm-van Mil, A. H.; Cerami, A.; Huizinga, T. W.; Toes, R. E.; Trouw, L. A. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc. Natl. Acad. Sci. USA* **108:** 17372–17377; 2011.

[16] van Beers, J. J.; Willemze, A.; Stammen-Vogelzangs, J.; Drijfhout, J. W.; Toes, R. E.; Pruijn, G. J. Anti-citrullinated fibronectin antibodies in rheumatoid arthritis are associated with HLA-DRB1 shared epitope alleles. *Arthritis Res. Ther.* **14:**R35; 2012.

[17] De Rycke, L.; Nicholas, A. P.; Cantaert, T.; Kruithof, E.; Echols, J. D.; Vandekerckhove, B.; Veys, E. M.; De Keyser, F.; Baeten, D. Synovial intracellular citrullinated proteins colocalizing with peptidyl arginine deiminase as pathophysiologically relevant antigenic determinants of rheumatoid arthritis-specific humoral autoimmunity. *Arthritis Rheum.* **52:**2323–2330; 2005.

[18] Wang, Z.; Nicholls, S. J.; Rodriguez, E. R.; Kummu, O.; Horkko, S.; Barnard, J.; Reynolds, W. F.; Topol, E. J.; DiDonato, J. A.; Hazen, S. L. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat. Med* **13:**1176–1184; 2007.

[19] Donnelly, S.; Roake, W.; Brown, S.; Young, P.; Naik, H.; Wordsworth, P.; Isenberg, D. A.; Reid, K. B.; Eggleton, P. Impaired recognition of apoptotic neutrophils by the C1q/calreticulin and CD91 pathway in systemic lupus erythematosus. *Arthritis Rheum.* **54:**1543–1556; 2006.

[20] Morgan, P. E.; Sturgess, A. D.; Hennessy, A.; Davies, M. J. Serum protein oxidation and apolipoprotein CIII levels in people with systemic lupus erythematosus with and without nephritis. *Free Radic. Res.* **41:**1301–1312; 2007.

[21] Kurien, B. T.; Porter, A.; Dorri, Y.; Iqbal, S.; D'Souza, A.; Singh, A.; Asfa, S.; Cartellieri, M.; Mathias, K.; Matsumoto, H.; Bachmann, M.; Hensley, K.; Scofield, R. H. Degree of modification of Ro60 by the lipid peroxidation by-product 4-hydroxy-2-nonenal may differentially induce Sjogren syndrome or systemic lupus erythematosus in BALB/c mice. *Free Radic. Biol. Med.* **50:**1222–1233; 2011.

[22] Scofield, R. H.; Kurien, B. T.; Ganick, S.; McClain, M. T.; Pye, Q.; James, J. A.; Schneider, R. I.; Broyles, R. H.; Bachmann, M.; Hensley, K. Modification of lupus-associated 60-kDa Ro protein with the lipid oxidation product 4-hydroxy-2-nonenal increases antigenicity and facilitates epitope spreading. *Free Radic. Biol. Med.* **38:**719–728; 2005.

[23] Wang, G.; Pierangeli, S. S.; Papalardo, E.; Ansari, G. A.; Khan, M. F. Markers of oxidative and nitrosative stress in systemic lupus erythematosus: correlation with disease activity. *Arthritis Rheum.* **62:**2064–2072; 2010.

[24] Griffiths, H. R. Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? *Autoimmun. Rev* **7:**544–549; 2008.

[25] Ling, S.; Li, Z.; Borschukova, O.; Xiao, L.; Pumpens, P.; Holoshitz, J. The rheumatoid arthritis shared epitope increases cellular susceptibility to oxidative stress by antagonizing an adenosine-mediated anti-oxidative pathway. *Arthritis Res. Ther.* **9:**R5; 2007.

[26] Moore, A. R.; Appelboam, A.; Kawabata, K.; Da Silva, J. A. P.; D'Cruz, D.; Gowland, G.; Willoughby, D. A. Destruction of articular cartilage by alpha-2-macroglobulin elastase complexes: role in rheumatoid arthritis. *Ann. Rheum. Dis.* **58:**109–113; 1999.

[27] Cavalleri, A.; Colombo, C.; Venturelli, E.; Miceli, R.; Mariani, L.; Cornelli, U.; Pala, V.; Berrino, F.; Secreto, G. Evaluation of reactive oxygen metabolites in frozen serum samples: effect of storage and repeated thawing. *Int. J. Biol. Markers* **19:**250–253; 2004.

[28] Ryan, B. (2011) The effect of oxidative post-translational modifications to C1q on its antigenicity and function, in relation to the pathogenesis of systemic lupus erythematosus. Plymouth, UK: Peninsula College of Medicine and Dentistry. [Ph.D. thesis].

[29] Siegert, C. E. H.; Daha, M. R; Tseng, C. M. E. S.; Coremans, I. E. M.; van Es, L. A.; Breedveld, F. C. Predictive value of IgG autoantibodies against C1q for nephritis in systemic lupus erythematosus. *Ann. Rheum. Dis.* **52:**851–856; 1993.

[30] Tsimikas, S.; Brilakis, E. S.; Lennon, R. J.; Miller, E. R.; Witztum, J. L.; Mcconnell, J. P.; Kornman, K. S.; Berger, P. B. Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. *J. Lipid Res.* **48:**425–433; 2007.

[31] Winyard, P. G.; Tatzber, F.; Esterbauer, H.; Kus, M. L.; Blake, D. R.; Morris, C. J. Presence of foam cells containing oxidised low density lipoprotein in the synovial membrane from patients with rheumatoid arthritis. *Ann. Rheum. Dis.* **52:**677–680; 1993.

[32] Svenungsson, E.; Jensen-Urstad, K.; Heimburger, M.; Silveira, A.; Hamsten, A.; de Faire, U.; Witztum, J. L.; Frostegard, J. Risk factors for cardiovascular disease in systemic lupus erythematosus. *Circulation* **104:**1887–1893; 2001.

[33] Toru, K.; Noriaki, K.; Manabu, M.; Kazutaka, H.; Toshinori, M.; Hideto, S.; Hideaki, M.; Hiroshi, K.; Eiichiro, N.; Hisanori, H.; Hidenori, A.; Masayuki, Y. Role of oxidized LDL in atherosclerosis. *Ann. N. Y. Acad. Sci.* **947:**199–206; 2001.

[34] Zaguri, R.; Verbovetski, I.; Atallah, M.; Trahtemberg, U.; Krispin, A.; Nahari, E.; Leitersdorf, E.; Mevorach, D. 'Danger' effect of low-density lipoprotein (LDL) and oxidized LDL on human immature dendritic cells. *Clin. Exp. Immunol.* **149:**543–552; 2007.

[35] Shaw, P. X.; Hörkkö, S.; Chang, M. -K.; Curtiss, L. K.; Palinski, W.; Silverman, G. J.; Witztum, J. L. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J. Clin. Invest.* **105:**1731–1740; 2000.

[36] Sukhanov, S.; Higashi, Y.; Shai, S. -Y.; Itabe, H.; Ono, K.; Parthasarathy, S.; Delafontaine, P. Novel effect of oxidized low-density lipoprotein: cellular ATP depletion via downregulation of glyceraldehyde-3-phosphate dehydrogenase. *Circ. Res.* **99:**191–200; 2006.

[37] Haugland, R. P. Antibody conjugates for cell biology. *Curr. Protoc. Cell Biol.* (Suppl. 6):16.5.1–16.5.22; 2000. Unit 16.5.

[38] Frears, E. R.; Zhang, Z.; Blake, D. R.; O'Connell, J. P.; Winyard, P. G. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. *FEBS Lett.* **381:**21–24; 1996.

[39] Winyard, P. G.; Lunec, J.; Brailsford, S.; Blake, D. Action of free radical generating systems upon the biological and immunological properties of caeruloplasmin. *Int. J. Biochem.* **16:**1273–1278; 1984.

[40] Kim, J.; Chehade, J.; Pinnas, J. L.; Mooradian, A. D. Effect of select antioxidants on malondialdehyde modification of proteins. *Nutrition* **16:**1079–1081; 2000.

[41] Kang, J. H. Modification and inactivation of human Cu,Zn-superoxide dismutase by methylglyoxal. *Mol. Cells* **15:**194–199; 2003.

[42] Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology Medicine.* Oxford: Oxford Univ. Press; 1999.

[43] Whiteman, M.; Szabo, C.; Halliwell, B. Modulation of peroxynitrite- and hypochlorous acid-induced inactivation of alpha1-antiproteinase by mercaptoethylguanidine. *Br. J. Pharmacol.* **126:**1646–1652; 1999.

[44] Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87:**1620–1624; 1990.

[45] Hughes, M. N.; Nicklin, H. G. The chemistry of pernitrites. Part I. Kinetics of decomposition of pernitrous acid. *J. Chem. Soc. A* :450–452; 1968.

[46] Whiteman, M.; Kaur, H.; Halliwell, B. Protection against peroxynitrite dependent tyrosine nitration and alpha-1-antiproteinase inactivation by some anti-inflammatory drugs and by the antibiotic tetracycline. *Ann. Rheum. Dis* **55:**383–387; 1996.

[47] Fabisiak, J. P.; Sedlov, A.; Kagan, V. E. Quantification of oxidative/nitrosative modification of CYS(34) in human serum albumin using a fluorescence-based SDS–PAGE assay. *Antioxid. Redox Signaling* **4:**855–865; 2002.

[48] Barnes, S.; Shonsey, E. M.; Eliuk, S. M.; Stella, D.; Barrett, K.; Srivastava, O. P.; Kim, H.; Renfrow, M. B. High-resolution mass spectrometry analysis of protein oxidations and resultant loss of function. *Biochem. Soc. Trans.* **36:**1037–1044; 2008.

[49] Spickett, C. M.; Pitt, A. R. Protein oxidation: role in signalling and detection by mass spectrometry. *Amino Acids* **42:**5–21; 2012.

[50] Zendman, A. J.; van Venrooij, W. J.; Pruijn, G. J. Use and significance of anti-CCP autoantibodies in rheumatoid arthritis. *Rheumatology (Oxford)* **45:**20–25; 2006.

[51] Leeb, B. F.; Andel, I.; Sautner, J.; Nothnagl, T.; Rintelen, B. The DAS28 in rheumatoid arthritis and fibromyalgia patients. *Rheumatology (Oxford)* **43:**1504–1507; 2004.

[52] Pope, J. The revised BILAG Index with numerical scoring in systemic lupus erythematosus: added value with some limitations. *Rheumatology (Oxford)* **49:**1616–1617; 2010.

[53] Bombardier, C.; Gladman, D. D.; Urowitz, M. B.; Caron, D.; Chang, C. H. Derivation of the SLEDAI: a disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum.* **35:**630–640; 1992.

[54] Neogi, T.; Aletaha, D.; Silman, A. J.; Naden, R. L.; Felson, D. T.; Aggarwal, R.; Bingham 3rd C. O.; Birnbaum, N. S.; Burmester, G. R.; Bykerk, V. P.; Cohen, M. D.; Combe, B.; Costenbader, K. H.; Dougados, M.; Emery, P.; Ferraccioli, G.; Hazes, J. M.; Hobbs, K.; Huizinga, T. W.; Kavanaugh, A.; Kay, J.; Khanna, D.; Kvien, T. K.; Laing, T.; Liao, K.; Mease, P.; Menard, H. A.; Moreland, L. W.; Nair, R.; Pincus, T.; Ringold, S.; Smolen, J. S.; Stanislawska-Biernat, E.; Symmons, D.; Tak, P. P.; Upchurch, K. S.; Vencovsky, J.; Wolfe, F.; Hawker, G. The 2010 American College of Rheumatology/European League against Rheumatism classification criteria for rheumatoid arthritis: phase 2 methodological report. *Arthritis Rheum* **62:**2582–2591; 2010.

[55] Stoll, T.; Stucki, G.; Malik, J.; Pyke, S.; Isenberg, D. A. Association of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index with measures of disease activity and health status in patients with systemic lupus erythematosus. *J. Rheumatol* **24:**309–313; 1997.