Is the generation of neoantigenic determinants by free radicals central to the development of autoimmune rheumatoid disease?

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Abstract

Biomolecules are susceptible to many different post-translational modifications that have important effects on their function and stability, including glycosylation, glycation, phosphorylation and oxidation chemistries. Specific conversion of aspartic acid to its isoaspartyl derivative or arginine to citrulline leads to autoantibody production in models of rheumatoid disease, and ensuing autoantibodies cross-react with native antigens. Autoimmune conditions associate with increased activation of immune effector cells and production of free radical species via NADPH oxidases and nitric oxide synthases. Generation of neo-antigenic determinants by reactive oxygen and nitrogen species ROS and RNS) may contribute to epitope spreading in autoimmunity. The oxidation of amino acids by peroxynitrite, hypochlorous acid and other reactive oxygen species (ROS) increases the antigenicity of DNA, LDL and IgG, generating ligands for which autoantibodies show higher avidity. This review focuses on the evidence for ROS and RNS in promoting the autoimmune responses observed in diseases rheumatoid arthritis (RA) and Systemic Lupus Erythematosus (SLE). It considers the evidence for ROS/RNS-induced antigenicity arising as a consequence of failure to remove or repair ROS/RNS damaged biomolecules and suggests that an associated defect, probably in T cell signal processing or/or antigen presentation, is required for development of disease.

Keywords – reactive oxygen species; reactive nitrogen species; protein oxidation; autoantigen; neoantigenic determinant; DNA oxidation

Take home messages;

- Free radical modification of IgG exposes neo-antigenic determinants and generates an antigen with higher avidity of interaction with rheumatoid factor than native IgG.
- Free radical modification of DNA exposes neo-antigenic determinants and generates an antigen with high avidity of interaction with anti-ds DNA autoantibodies than native DNA.
- Failure to remove/repair modified antigens appear may be important for the presentation of neoantigenic determinants to T cells.
- Absence of autoimmunity in other diseases where autoantibodies are detected e.g. cancer suggests that dysfunctional antigen presentation must also be important in driving the aberrant inflammatory response.

The phenotype of diffuse autoimmune diseases can be attributed, at least in part, to abnormalities of the T cell population with an increased prevalence of $CD4^{+}CD28^{null}$ T cells which are neither anergic nor functionally paralyzed [1]. These cells have gained proinflammatory capacities and cytotoxic function and are resistant to apoptosis; T cell dysfunction is further confounded in autoimmunity by the presence of persistent immune complexes arising from the failure of effective antigen removal. Subsequent interaction with antibody results in immune complex formation and in turn the complexes bind phagocytic cells via Fcγ receptors to facilitate their clearance. Receptor engagement triggers a cascade of intracellular pathways in effector cells resulting in production of free radicals by the NADPH oxidase on infiltrating neutrophils, monocyte/macrophages and resident tissue macrophages with concomitant activation of NFkB and pro-inflammatory gene expression [2]. The formation of high levels of the nitrogen-centred free radical, nitric oxide, in macrophages via iNOS in humans is debated although recent evidence from SLE patients suggests increased nitrite production associated with inflammation [3]. The release of myeloperoxidase from neutrophilic granules following activation by immune complexes also contributes to the oxidative environment in autoimmune disease [4]. Whilst low levels of oxidants have important roles as signalling molecules, over-production in the absence of adequate antioxidant defence may cause irreversible changes to biomolecules and contribute to disease progression.

Rheumatoid arthritis (RA) has a prevalence of 1:3 of the over-65 population of developed countries, with both systemic and localised (within articular joints) components. The rheumatoid joint is recognised as a site which undergoes repetitive cycles of ischemia and reperfusion and formation of oxygen-derived free radicals [5] with the gene expression profile varying according to the degree of hypoxia and re-

oxygenation. The autoimmune disease, systemic lups erythematosus (SLE), has a strong inflammatory component, which is typified by chronic over-production of ROS and RNS [6].

This review focuses on the current knowledge of the differential roles of acute and chronic ROS and RNS production in the generation of neo-antigenic determinants which may serve as drivers of T and /or B cell activation with particular focus on SLE and RA.

Generation of neo-antigenic determinants

Why antigens which have previously been tolerated may later be seen as foreign is the subject of several hypotheses; molecular mimicry has recently been reviewed by Blank [7] and is based on the concept that viral or bacterial antigens serve as antigenic mimics, i.e., identical to a self protein and, although a normal immune response is developed to the pathogen, the resultant antibodies show cross-reactivity towards self-antigen.

More recently, epitope spreading has gained credence as a major driver underlying autoimmunity (Figure 1). This hypothesis suggests that the development of antibodies against other cryptic epitopes on the original antigen, after self-tolerance has been broken, provides a mechanism for development of autoantibody production in a number of autoimmune diseases including SLE [8]. This model is supported by experimental development of autoimmunity in non-autoimmune mice following immunisation with self-peptides containing cryptic sites [9]; post-translational modification of proteins may elicit an immune response which shows cross-reactivity with native peptides.

Evidence is accumulating for induction of autoimmune responses after a number of different post-translational modifications within target proteins [9]; while proteins are

normally encoded by variable sequences of 20 amino acids, there are hundreds of modified forms of amino acids which may be incorporated into the protein sequence [10]. Such modifications can contribute to changes in the backbone [11], alterations in charge [12] and differential 3-dimensional folding pattern [13]. Any of these changes may generate neo-antigenic determinants, and frequently antibodies raised against peptides with minor structural modification can also show cross-reactivity with the native protein, thus exhibiting true autoreactivity with self [10]. Neo-antigenic determinants may also arise from environmental damage such as UV exposure [14] and as a side effect from therapeutic drugs, such as procainamide [14], which can lead to adduct formation with self-antigens.

The detection of autoantibodies against citrullinated proteins, particularly of extracellular matrix proteins such as collagen, improves diagnostic specificity for RA when compared with rheumatoid factor [15], but there is no clear evidence for increased deimination under oxidative stress.

Another important post-translational modification of proteins is the non-enzymatic conversion of aspartate to isoaspartic acid [16]. This process occurs spontaneously at a low rate, but is enhanced under conditions of stress, exposure to the oxidant hydrogen peroxide and with ageing [17]. Mamula et al. [18] reported that autoimmune responses characteristic of SLE can be elicited by immunisation with isoaspartyl forms of small nuclear (sn) ribonuclear proteins. In addition, Yang et al. [19] observed increased intracellular levels of isoaspartyl residues in T cell proteins from SLE mice and this has been suggested to contribute to hyperreactivity. Isoaspartyl residues are subject to repair by the intracellular repair enzyme, protein isoaspartyl methyl transferase (PIMT), which can restore normal aspartyl configuration. PIMT deficient mice express

increased levels of anti-DNA antibodies typical of SLE indicating the importance of PIMT activity for maintaining immune tolerance [20]. The activity of this enzyme is not influenced by ROS despite increased formation of isoaspartyl residues in erythrocytes after exposure to hydrogen peroxide [17]. This suggests that damage occurs in excess of the capacity to repair, rather than as a deficiency in the repair of post-translational modifications may be an important contributor to autoantigen accumulation.

ROS and RNS levels are increased in autoimmune diseases such as RA and SLE. The over-production of ROS/RNS may exceed the capacity for radical scavenging by antioxidant enzymes or small inhibitors and may modify all classes of biomolecules [21] therefore generating or exposing neo-antigenic determinants on biomolecules which perpetuate antigen driven autoimmune responses [22]. Exposure of proteins to ROS and RNS alters their composite amino acids, according to the radical species and the protein structure. However, oxidative damage to biomolecules is rarely specific and is dependent on the concentration of the protein, its cellular location with respect to cellular oxidant generating systems and the rate of modified protein clearance. Increased oxidation of LDL also has been reported in RA, with the appearance of foam cell-like structures within the rheumatoid synovium [12, 23]. Recent studies have confirmed that vascular disease is prevalent in RA, with greater than 50% of RA deaths being attributed to cardiovascular disease [24].

In addition to the prevalent oxidative reactions in RA, Kaur and Halliwell have demonstrated a substantial augmentation in tyrosine nitration of plasma proteins is also associated with RA [25] and similar observations of elevated 3-nitrotyrosine levels which correlated with disease activity were later reported in SLE [26]. A more comprehensive analysis of protein oxidation recently has been undertaken in SLE and has shown

elevated levels of protein carbonyls, methionine sulphoxide and 3-nitrotyrosine in serum proteins with a corresponding reduction in protein thiol content [27] confirming the frequency of post-translational protein modifications in autoimmunity.

Although proteins are the most common antigens in autoimmune disease, SLE is characterised by the presence of autoantibodies to DNA, and there is mounting evidence for enhanced levels of DNA oxidation products, particularly 8-oxodeoxyguanine (8-oxo-dG), in SLE. Increased levels of oxidation of the base, guanine, to form 8-oxoguanine, is observed in the DNA within immune complexes from SLE patients compared with those from RA patients [28]. In addition, levels of 8-oxo-dG in the urine of SLE patients are significantly reduced [28]. These observations have led to the suggestion that aberrant DNA repair in SLE patients contributes to an accumulation of oxidised DNA exposed during apoptosis which can form immune complexes and thus perpetuate systemic inflammation [28].

Modification of antigens by ROS and RNS

Several authors have shown that IgG is susceptible to oxidative modification which may induce aggregation, amino acid modification and/or fragmentation which vary according to the denaturing species; hydroxyl radicals cause IgG aggregation, whereas peroxy radical species may induce chemical modification in the absence of aggregation [29]. In contrast, superoxide anion radicals, in catalytic metal ion free reactions, do not cause any biochemical or structural modification. Amino acid analysis has revealed the loss of a number of amino acids, which vary according to the denaturing species but include tryptophan, methionine and tyrosine. Using reversed phase HPLC, we confirmed that tryptophan was converted to N-formyl kynurenine and could be detected in rheumatoid serum IgG at higher levels than in serum IgG from age-matched controls

[30]. Correspondingly, rheumatoid factor shows higher avidity towards IgG which has been modified by OH• than native IgG suggesting that such oxidative modifications would perpetuate antigen driven immune responses against IgG [22]. Using UV radiation to generate radical on the protein backbone, aggregation and significant loss of tryptophan from IgG has been observed. These changes were shown to correspond with changes in IgG isolated from RA patients and an increase in antigenicity of IgG [30].

In addition to release of simple oxidants, activated neutrophils also release myeloperoxidase which catalyses the formation of HOCl from hydrogen peroxide and chloride ions. Jasin and colleagues have investigated the effects of HOCl on IgG and have demonstrated that formation of mercaptoethanol-resistant, Schiff-base crosslinks following the oxidation of lysine residues [4]. Moreover, HOCl or peroxynitrite treatment of IgG caused a dose dependent reduction in complement binding and activation, and a decrease in binding to Fc receptors on monocytes [31]. The functional changes reported above suggest a pro-inflammatory effect, but HOCl modified or nitrated IgGs were found to be 20-fold less effective than native IgG in inducing acute inflammation in synovial joints, indicating a complex physiological response to IgG chlorination or nitration [32].

Antibodies against oxidised LDL have been being reported in both SLE and RA [33]. Indeed, patients with early RA have elevated auto-antibody titres against oxidised LDL compared to healthy controls [34]. Increased levels of autoantibodies towards nitrated LDL in RA patients with cardiovascular complications have been observed where nitrated LDL was more avidly scavenged by macrophages than native or oxidised LDL [35]. The clearance of oxidised or nitrated LDL via scavenger receptors such as CD36 present on macrophages may contribute to the incidence of cardiovascular complications frequently observed in RA patients.

Advanced glycation end-product modifications to proteins are common in diabetes, but have also is observed in other conditions such as RA [36]. Correspondingly, autoantibodies are observed to AGE-IgG. The mechanisms underlying the frequency of this modification in RA are unclear. However, the absence of hyperglycaemia in RA patients suggests an accumulation of AGE-modified protein due to failure in degradative pathways rather than increased production of AGE as would be expected in diabetes.

Many of the oxidative modifications of proteins *in vitro* cause their aggregation, thus increasing the valency of a given antigen for antibody. In addition, oxidation causes conformational changes including that exposes the hydrophobic regions of proteins, increasing their likelihood to bind to other hydrophobic moieties and therefore their propensity for degradation [37]; both of these characteristics may explain the increased interaction between autoantibodies and autoantigens after oxidation.

We previously have shown that oxidation of DNA produces an antigen that is more discriminating for antibodies present in SLE sera than native DNA and shows increased avidity of antibody binding [38]. More recent studies have investigated the effects of peroxynitrite on DNA antigenicity; Habib et al. reported that autoantibodies to DNA from SLE patients recognised peroxynitrite-modified DNA better than the native DNA molecule [39]; these data support the argument that induction of neoantigenic determinants in DNA after modification by nitric oxide may induce high titre immunogen-specific antibodies that cross-react with polynucleotides and nucleic acid.

The oxidation of lipids, particularly polyunsaturated fatty acids, by ROS and RNS is widely reported. Lipids exhibit conformational flexibility and thus are only weakly antigenic, but their oxidation can lead to the formation of a number of reactive intermediates, including 4-hydroxynonenal and malondialdehyde, which may

subsequently modify other macromolecules, including proteins via the E-amino group of lysine and DNA. Scofield et al. have demonstrated that 4-hydoxynonenal-mediated modification of the lupus-associated 60KDa Ro protein, which associates with RNA, increases its antigenicity and facilitates epitope spreading to the nuclear antigens La, dsDNA and snRNP [34].

The foregoing evidence supports the involvement of oxidatively damaged biomolecules as antigens which have high avidity for antibodies in the perpetuation of autoimmune disease. Indeed, the importance of neo-antigenic determinants in driving autoantibody formation is confirmed by the prevalence of elevated levels of autoantibodies against DNA, particularly for oxidised DNA [40]. That cancer patients do not develop SLE supports the hypothesis that there may be additional aberrations in the control of the immune system e.g. in antigen presentation and T cell dysfunction, which predispose to the development of autoimmune diseases, particularly in the elderly.

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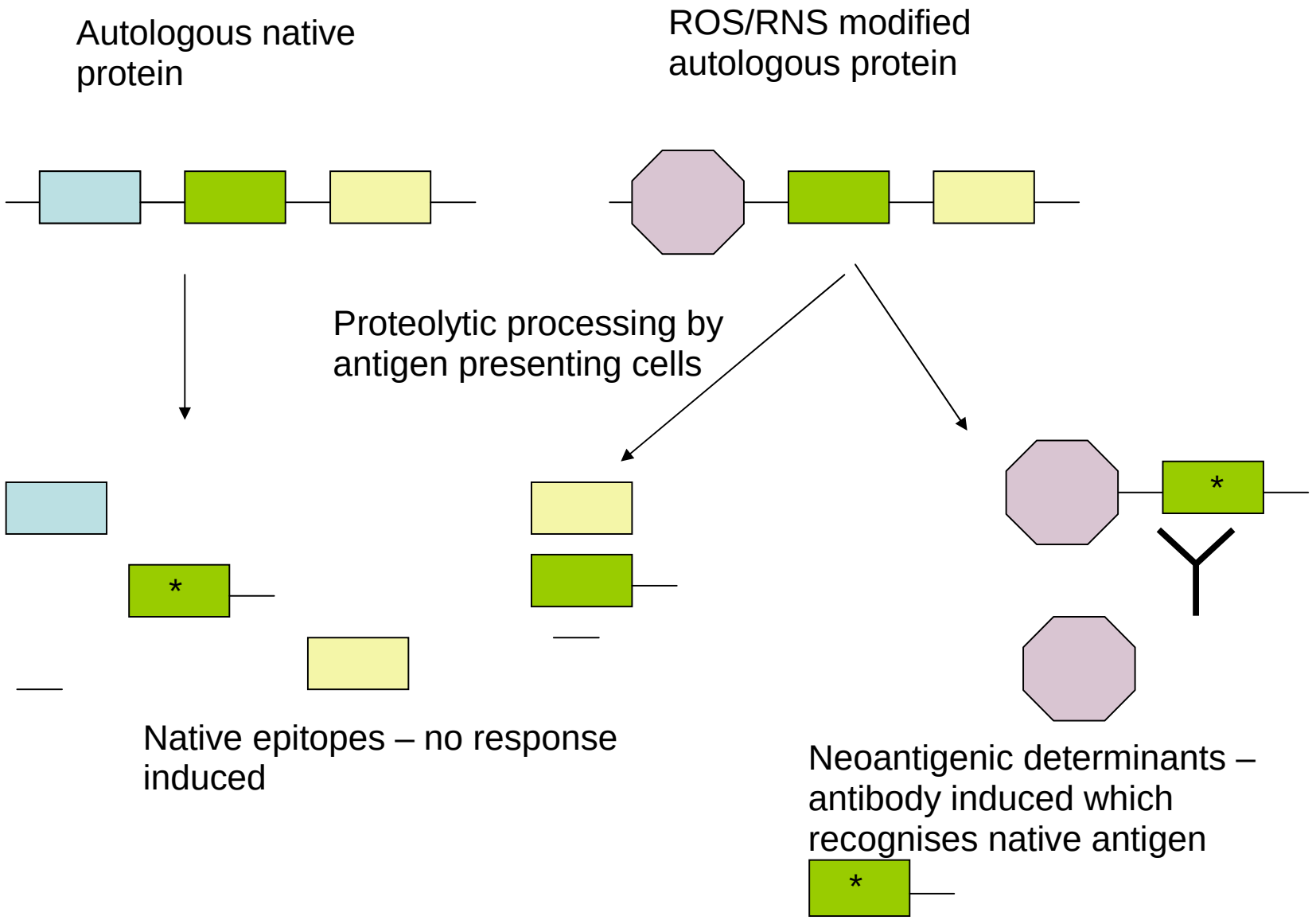
Legends

Figure 1. **Neoantigenic determinant-driven antibody response**

Native peptides do not trigger immune responses. Modification of amino acids by reactive oxygen species (ROS) or reactive nitrogen species (RNS) may alter proteolytic processing revealing new epitopes which are immunogenic. Antibodies reactive with neo-antigenic determinants may subsequently cross-react with native antigens in a process know as epitope spreading.

Figure 3


Autologous native protein
ROS/RNS modified autologous protein
Proteolytic processing by antigen presenting cells
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Native epitopes – no response induced
Neoantigenic determinants – antibody induced which recognises native antigen
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