

Redox-Reactive Autoantibodies: Biochemistry, Characterization, and Specificities

John A. McIntyre · W. Page Faulk

© Humana Press Inc. 2008

Abstract Oxidation–reduction (redox) reactions can “unmask” autoantibody activity in blood and other body fluids from normal, healthy individuals. These “unmasked” autoantibodies are similar if not identical to autoantibodies associated with autoimmune diseases. The agents responsible for this unmasking are physiological oxidants such as hemin and likely other naturally occurring molecules in the body that contain transitional metals available for participation in redox reactions. Laboratory comparisons between oxidized and non oxidized IgG fail to show differences to account for the oxidation-induced alteration of antibody specifics. The autoantibodies unmasked by redox reactivities represent a growing list of specificities, many that are responsible for modulating and/or regulating intracellular functions. In contrast, alloantibodies, such as anti-HLA antibodies, do not exhibit susceptibility to oxidation-induced autoantibody alterations, suggesting differences in the amino acids responsible for forming the complementarity determining regions of these respective antibody molecules. We have proposed that such reversible oxidative

conversions of antibody reactivities represent a heretofore undiscovered, but an evolutionary-conserved, resource of innate humoral immunity destined to maintain an immunological homeostasis.

Keywords Redox-reactive autoantibodies · Biochemistry · Characterization · Specificities · Oxidation–reduction reaction · Unmasked autoantibody activity

Background

Antibodies are proteins found circulating in the blood and other body fluids. One function of antibodies is to protect the “self” from harm by foreign “antigens” that enter the body such as bacteria and viruses. Antibodies bind to the foreign antigens and then signal other components of the immune system to destroy the “invader.” However, certain antibodies bind to the body’s own (“self”) tissues and cause a damaging inflammatory response. These antibodies are called autoantibodies [1].

In a very simplified model of the human immune system, there are two types of antibody responses: alloantibody and autoantibody. Alloantibodies are those that are produced in response to invading microbes, viruses, and other foreign structures, such as those in transplanted tissues. This type of antibody is usually protective of the health of the individual, transplant rejection notwithstanding. Autoantibodies produced to self-antigens can play a beneficial role in the removal of aged and dying cells within the body. In other instances, however, autoantibodies may attack healthy self-tissues in the body, causing an autoimmune disease. This paradoxical and potentially very dangerous clinical situation was recognized and named “horror autotoxicus” more than 100 years ago [2].

J. A. McIntyre (✉) · W. P. Faulk
HLA-Vascular Biology Laboratory,
St. Francis Hospital and Health Care Centers,
1600 Albany Street, Suite 1109,
Beech Grove, IN 46107, USA
e-mail: john.mcintyre@ssfhs.org

J. A. McIntyre
Department of Biology,
Indiana University–Purdue University Indianapolis,
Indianapolis, IN 46202, USA

W. P. Faulk
Faulk Pharmaceuticals, Inc.,
125 Hampton River Club Marina Drive,
St. Simons Island, GA 31522, USA

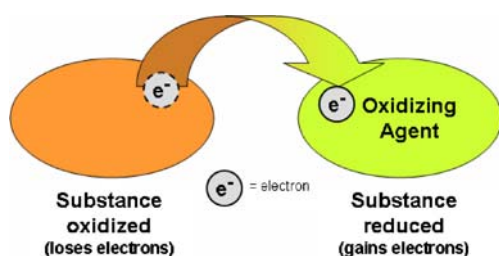


Fig. 1 Oxidation–reduction (redox) reaction

Normally, the autoantibodies in an individual are kept in a healthy balance or homeostasis. When homeostasis is disrupted, the body can, in effect, attack itself and cause a variety of autoimmune disorders, such as diabetes, lupus, rheumatoid arthritis, and a host of others. Over 100 autoimmune disorders have been identified in humans [3]. Numerous animal species (dogs, cats, horses, etc.) also suffer from autoimmune disorders [4].

We have discovered that oxidation “unmasks” antibodies in the blood and other body fluids from normal, healthy individuals revealing autoantibodies that are associated with autoimmune disorders. Conversely, oxidation of autoantibodies from individuals with autoimmune diseases can also cause remasking of their autoantibodies which then become undetectable. These conversions depend upon oxidation–reduction reactions and define a new family of autoantibodies that has been designated as redox-reactive autoantibodies (R-RAA).

A brief oxidation–reduction primer

Oxidation–reduction reactions are commonly known as “redox” reactions. As shown in Fig. 1, one molecule acts as an “oxidizer” by taking an electron(s) from a second molecule, which is then “oxidized” due to the loss of the electron(s).

We use redox reactions to cause an autoantibody to become detectable by unmasking previously masked autoantibodies. A practical example of this process (Fig. 2) is found when a commonly studied group of autoimmune antibodies called antiphospholipid antibodies (aPL) are unmasked in normal aPL-negative blood by exposure to a physiological oxidizing agent known as hemin, which is a normal product of the metabolism of hemoglobin. The oxidizing agent can be a molecule such as hemin or permanganate or it can be an electromotive force applied via a direct electrical current across two electrodes. In the cartoon in Fig. 2, the antibody, such as aPL, is generically indicated by IgG (i.e., immunoglobulin G).

Conformation of the transitional metal iron (Fe) as the responsible element in the hemin molecule was obtained by

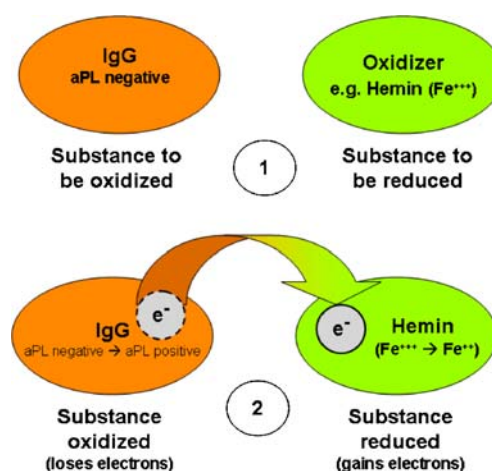


Fig. 2 A cartoon to demonstrate the oxidation of IgG by the oxidizing agent, hemin

comparisons between hemin and its noniron-containing congener, as shown in Fig. 3.

Discovery of R-RAA

R-RAA were first identified in blood, cerebrospinal fluid (CSF), and breast milk of all healthy individuals tested. [5, 6] R-RAA of G, M, and A isotypes exist in all animals tested to date (horses, dogs, mice, and IgY for chickens) and are likely found in all vertebrates. Studies have shown that CSF from normal individuals is limited to IgG, whereas breast milk is primarily IgA. Blood contains all three isotypes. A search for R-RAA of IgE and IgD isotypes was unsuccessful (unpublished data).

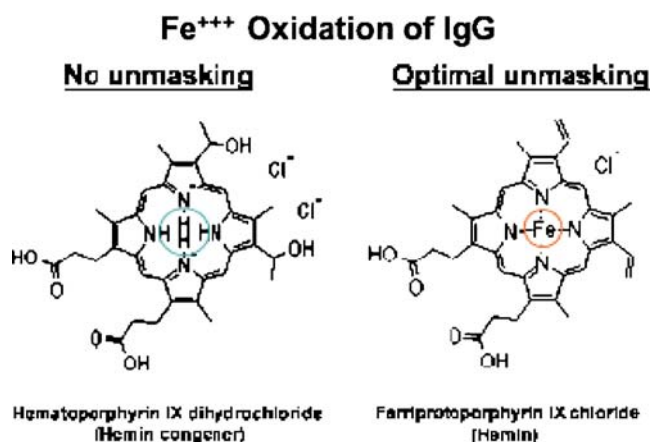


Fig. 3 Shown are two molecules that differ by the presence or absence of an iron atom depicted in the molecule on the right. The iron atom is transitional, i.e., it can be an electron donor or acceptor and thus participate in redox reactions. Only the molecule containing transitional iron is associated with unmasking of aPL autoantibodies. No unmasking of aPL autoantibody reactivity was demonstrated in the presence of the congener shown on the left

Table 1 Listing of redox-reactive autoantibodies detected to date

Antiphospholipid antibodies:	Anti-PF4/heparin complex
aPS, aCL, aPE, aPC, LA	Anti-β2-glycoprotein I
Glutamic acid decarboxylase (GAD)	Anti-RBC (undefined)
Tyrosine phosphatase (IA-2)	SmRNP
Antinuclear antibodies (ANA)	Ro/SS-A
Anticell organelles:	La/SS-B
nucleolus, lamin, golgi	Scl-70
Antigranulocytes:	Jo-1
neutrophils, monocytes	Ribo-P
Anti-B lymphocyte	Oxidized LDL
Antimyeloperoxidase	Ro52
Antitumor cell lines:	PCNA
Raji, Jurkat, U87MG, K562	PmScl-100
Antitrophoblast (TBM)	Ku
Antifactor VIII	Mi-2

Our initial R-RAA discoveries involved the specific group of autoantibodies known as aPL. These autoantibodies target particular molecules in the membranes of cells, phospholipids, and/or plasma proteins bound to the phospholipids [7]. Using the aPL enzyme-linked immunosorbent assay (ELISA), we demonstrated that aPL-negative samples of human blood can be converted to IgG, IgM, and IgA aPL-positive samples by application of a redox reaction. In later studies, we began using IgG fractions as opposed to whole blood, as this simplified our understanding of the experimental model.

While much of the work has been focused on the aPL family of autoantibodies, Table 1 shows a list of redox-reactive autoantibodies that have been detected to date in a wide variety of assay conditions, including ELISA, radioimmunoassay (RIA), flow cytometry, Western blots, and immunofluorescence that were performed by investigators in several different commercial and research laboratories. This list is expected to continue to grow, especially in view

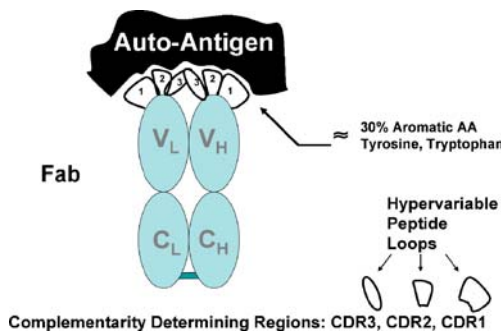


Fig. 4 Shown is the antibody binding fragment of an IgG molecule and the location of the hypervariable region that contains the CDR. It is the CDR that makes the interface between the autoantibody and the autoantigen

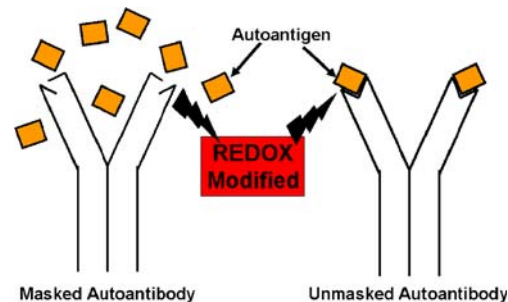


Fig. 5 Simplified visualization of the antibody-altering effect of the redox reaction to result in unmasking of autoantibody activity

of the proteomic (microarray) analyses recently performed at Invitrogen’s laboratories.

Data show that hemin can reversibly oxidize protein molecules and cause nitrosylation of tyrosine residues [8]. We have proposed nitrosylation as one mechanism to account for the altered specificity of an antibody’s hyper-variable or complementarity determining regions (CDR), since these antigen-binding sites contain selectively more conserved aromatic amino acids with redox potential than what would be expected were they selected at random [9]. A cartoon of the CDR is shown in Fig. 4. Another cartoon of the effect of oxidation upon the antibody molecule is shown in Fig. 5.

The discovery of R-RAA presents a new perspective wherein the immunological systems of human beings and many other vertebrates contain many autoantibodies that remain in a masked state. Unmasking will occur when there is a stimulus that produces an oxidizing agent or when reactive oxygen species are generated due to inflammation and/or infection.

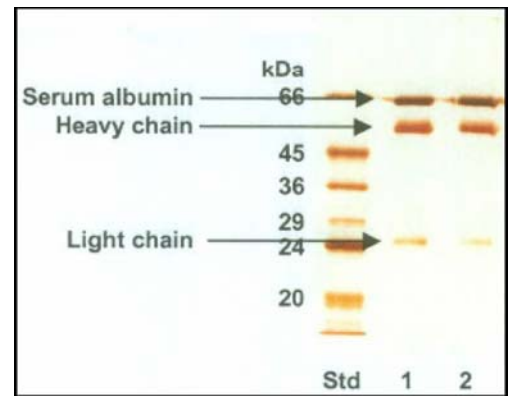


Fig. 6 SDS gel analysis shows identical nonoxidized IgG and R-RAA IgG profiles. A representative SDS-PAGE experiment performed on pharmaceutical-grade fractionated IgG shows that the redox-reacted sample (lane 2) cannot be differentiated from the native (lane 1) sample. Lanes 1 and 2 both show albumin (added as a stabilizer). Exposure to oxidation (lane 2) does not alter the electrophoretic properties of the IgG antibodies

We propose that some autoimmune diseases may result from the unmasking of these autoantibodies, and owing to a faulty redox reaction-driven mechanism, lack the ability to remask after unmasking. The persistence of R-RAA would suggest that they are important components of an innate humoral defense mechanism. Without a preceding redox reaction, R-RAA cannot be detected by using *in vitro* assays that depend upon antibody binding. This clearly differentiates them from the “natural” and “hidden” autoantibodies previously described by others [10–12]. To date, every human individual tested has been shown to possess this family of R-RAA.

Experiments have been performed to determine if oxidation of a purified fraction of IgG would cause measurable differences in the molecular migratory profiles of IgG before and after oxidation, and the results indicate no differences. For example, a reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiment of oxidized and not oxidized IgG revealed no differences, as shown in Fig. 6.

Absorption studies of R-RAA specificity

One of the many autoantibody reactivities observed by oxidizing a pharmaceutical IgG preparation revealed the presence of autoantibodies to glutamic acid decarboxylase

(GAD), which are identical to the autoantibodies found in patients with type 1 diabetes. Absorption studies of control and oxidized sera from five individuals and including two different R-RAA were performed by using a recombinant form of GAD versus an irrelevant protein to determine which removed more counts per minute (cpm) from a radioimmunoassay for anti-GAD activity. The results showed that GAD65 specifically removed cpm, indicating that the R-RAAs were of equivalent specificity as the anti-GAD autoantibodies found in diabetic sera. These data are presented in Fig. 7.

Analytical results from Invitrogen's Protein Microarray Protoarray®, a new tool for profiling antibody reactivity

The penultimate experiment for R-RAA antigen recognition characterization was arranged with Dr. Paul Predki of Invitrogen (Carlsbad, CA, USA) to use Invitrogen's Protein Microarray technology, PROTOARRAY®, to test the effect of hemin oxidation on a pharmaceutical preparation of IVIg.

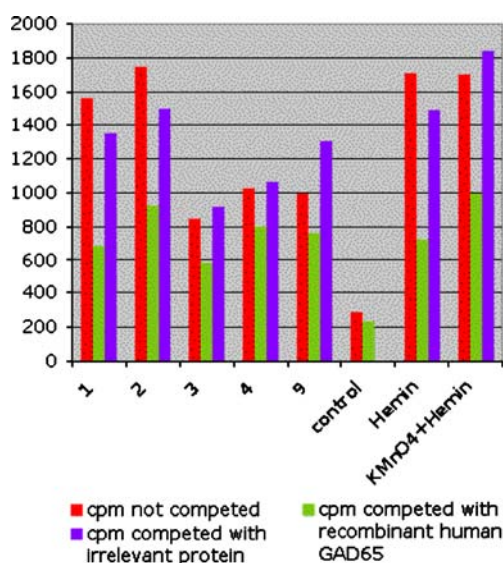


Fig. 7 Specificity of R-RAA anti GAD65 shown by radioimmunoassay. Bar graphs of radiolabeled antiglutamic acid decarboxylase (GAD65) RIA. Hemin was used as the oxidizing agent for oxidized plasma IgG from different individuals 1–4, 9, and for an IgG fraction (to the right of the control bars which represent nonoxidized IgG). Using two oxidizing agents, hemin+KMnO₄ did not increase reactivity over that achieved by using hemin only

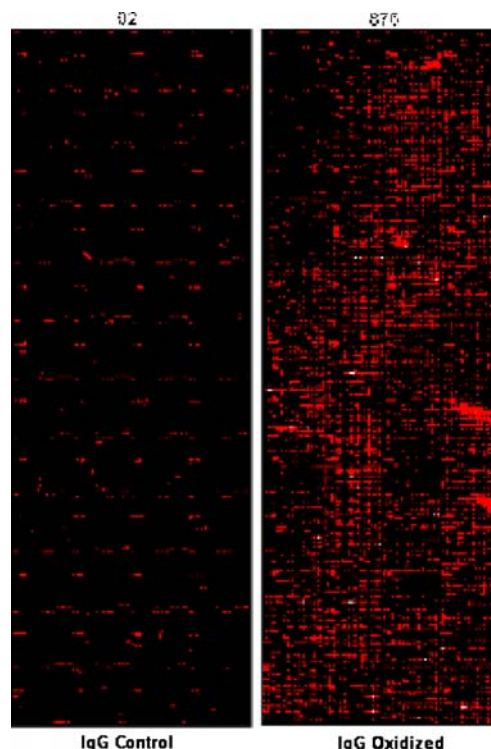


Fig. 8 The left panel shows the reactivity of the untreated IgG pool (control). The right panel shows the reactivity of the IgG after redox treatment with hemin. The dramatic difference between the untreated and oxidized IgG is likely partly due to the large number of individuals represented in the IgG pool

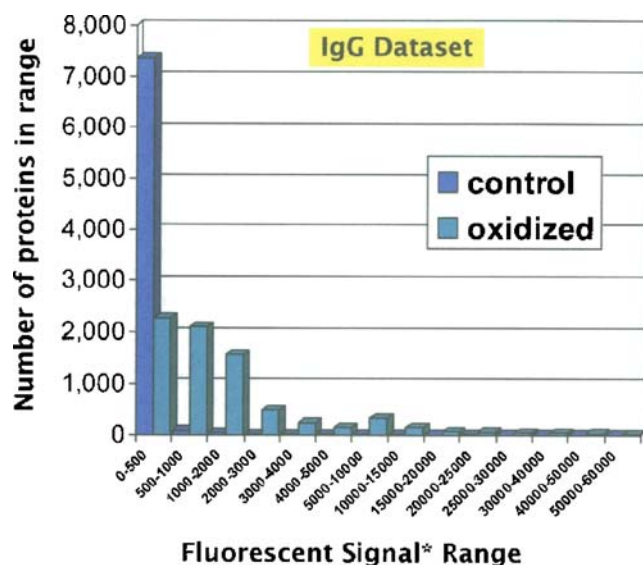


Fig. 9 The intensity of the signals shift dramatically after oxidation of IgG. The darker column shows that over 90% of the nontreated IgG had a low fluorescence intensity ranging from 0 to 500 fluorescent units. The green-blue bars represent the fluorescence intensities found with hemin-oxidized IgG

These arrays are often used for analysis (biomarker discovery) of autoantibodies in serum. The experiment was designed to run the treated and untreated samples on four arrays each containing 8,268 glutathione-*S*-transferase (GST) fusion human proteins, expressed in a baculoviral system. The proteins are dotted on the slide in duplicate on different areas of the slide, then fluorescence intensity of the duplicates is compared by correlation coefficients. During analyses, data are discarded for proteins that have disparate fluorescence intensities between the duplicate spots.

As primary antibodies, the IVIg sample was used at 50 $\mu\text{g/mL}$. The experimental protocol is shown below. After incubation of the primary antibody on the protein

dots, slides were washed and a secondary antibody (fluoresceinated antihuman IgG) was added. After washing and drying the slide, it was scanned on a fluorescent slide scanner, and after digitizing, reactive proteins were visually identified.

Figure 8 shows an overview of the technology/methodology. Experimental results are summarized in Figs. 9 and 10. The actual analysis and certain analytical comments in the figures were provided by Dr. Jeff Rogers, Senior Scientist for Invitrogen. The data graphs were constructed by Invitrogen.

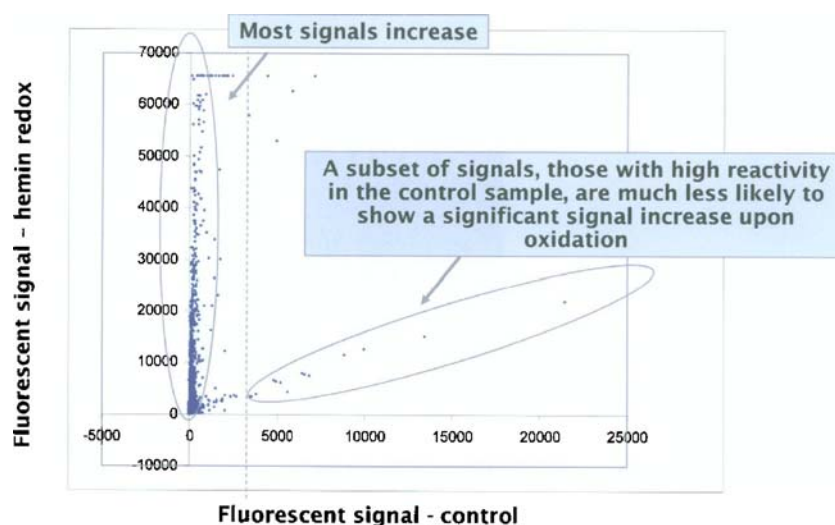
In examining the antigens with the highest fluorescent signal recognized by the hemin-treated IgG, i.e., R-RAA, in the ProtoArray[®], over 70% were kinases (phosphotransferases) and the other 30% were a mixture of signal transduction proteins, histone-related proteins, topoisomerases, zinc finger motif proteins, and others.

In contrast, the proteins which had the lowest fluorescent signals were many “housekeeping” proteins such as those involved with apoptosis, cytoskeleton (fibril and filament) and many nonkinase enzymes, DNA and RNA without binding proteins attached.

Development of atherosclerosis

In addition to type 1 diabetes, another common disease in which R-RAAs may have a role is atherosclerosis. In this disease, oxidation of low-density lipoproteins (LDL) initiate the generation of oxidation by-products [13]. These function in the early development of arterial lesions containing monocytes-derived macrophage foam cells filled with cholesteryl esters [14]. Oxidized low-density lipoproteins (oxLDL) bind different proteins, such as β_2 -glycoprotein I ($\beta_2\text{GPI}$), prothrombin, and C-reactive protein [15], and

Fig. 10 A different look at the IgG samples shows a bimodal effect of R-RAA binding to the proteins in the assay. An example of a control sample antibody that did not increase in fluorescence after hemin exposure was antibody to influenza A. A plausible explanation for such antibody reactivities that did not increase after oxidation is that they recognize alloantigens, not potential autoantibodies as exemplified on the x-axis



antibodies to oxLDL complexes have been identified in sera of patients with various autoimmune diseases, as well as in patients with cardiovascular diseases [16], but their significance is not clear. However, it is clear that IgG antibodies to oxLDL increase the in vitro macrophage uptake of oxLDL/ β_2 GPI complexes, suggesting a proatherogenic role for the antibodies [17]. In this regard, it is relevant that we have identified anti-oxLDL activity in hemin-mediated R-RAAs from normal blood donors (unpublished observations).

Finally, hemin itself may play a role in the generation of atherosclerosis lesions because hemin tightly binds to LDL with an association constant in the nanomoles per liter range, and such binding is not inhibited by other plasma proteins diluted to concentrations found in extracellular fluid where LDL oxidation is thought to occur [18]. In view of observations that hemin is both a powerful inducer of LDL oxidation and that hemin binds LDL, it has been implicated as functioning at an early stage in the development of atherosclerosis [19]. However, many molecules mediate physiological redox reactions, some of which have been implicated in different pathophysiological processes that are published in a recent forum by *Autoimmunity Reviews* [20].

Acknowledgements We thank Dr. Chinpal Kim, Purdue University, for Fig. 6 and Dr. Christiane Hampe, University of Washington, for Fig. 7. Mr. Napoleon Monce, Immunoconcepts, Sacramento, CA contributed to Table 1.

References

- Shoenfeld Y, Gershwin ME, Meroni PL (eds) (2008) *Autoantibodies*, 2nd edn. Elsevier, Philadelphia
- Silverstein AM (2001) Autoimmunity versus horror autotoxicus: the struggle for recognition. *Nat Immunol* 2:279–281
- National Institutes of Health, The Autoimmune Diseases Coordinating Committee (2005) Progress in autoimmune diseases research. Report to Congress. U.S. Department of Health and Human Services, March
- Gershwin LJ (2007) Veterinary autoimmunity: autoimmune diseases in domestic animals. *Ann N Y Acad Sci* 1109:109–116
- McIntyre JA (2004) The appearance and disappearance of antiphospholipid antibodies subsequent to oxidation–reduction reactions. *Thromb Res* 114:579–587
- McIntyre JA, Wagenknecht DR, Faulk WP (2005) Autoantibodies unmasked by redox reactions. *J Autoimmun* 24:311–317
- McIntyre JA, Wagenknecht DR, Faulk WP (2003) Antiphospholipid antibodies: discovery, definition, detection and disease. *Prog Lipid Res* 42:176–237
- McIntyre JA, Wagenknecht DR, Faulk WP (2006) Redox-reactive autoantibodies: detection and physiological relevance. *Autoimmun Rev* 5:76–83
- Fellouse FA, Barthelemy PA, Kelley RF, Sidhu SS (2006) Tyrosine plays a dominant functional role in the paratope of a synthetic antibody derived from a four amino acid code. *J Mol Biol* 357:100–114
- Cabiedes J, Cabral AR, Alarcón-Segovia (1998) Hidden antiphospholipid antibodies in normal human sera circulate as immune complexes whose antigen can be removed by heat, acid, hypermolar buffers or phospholipase treatments. *Eur J Immunol* 7:2108–2114
- Lorber M, Shoenfeld Y (2000) Hidden autoantibodies. *Clin Rev Allergy Immunol* 1:51–58
- Tomer Y, Shoenfeld Y (1988) The significance of natural autoantibodies. *Immunol Invest* 5:389–424
- Matsuura E, Hughes GRV, Khamashta MA (2008) Oxidation of LDL and its clinical implication. *Autoimmun Rev* 7:558–566
- Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320:915–924
- Tabuchi M, Inoue K, Usui-Kataoka H, Kobayashi K, Teramoto M, Takasugi K et al (2007) The association of C-reactive protein with an oxidative metabolite of LDL and its implication in atherosclerosis. *J Lipid Res* 48:768–781
- Shoenfeld Y, Wu R, Dearing LD, Matsuura E (2004) Are anti-oxidized low-density lipoprotein antibodies pathogenic or protective? *Circulation* 110:2552–2558
- Matsuura E, Kobayashi K, Tabuchi M, Lopez LR (2006) Oxidative modification of low-density lipoprotein and immune regulation of atherosclerosis. *Prog Lipid Res* 45:466–486
- Camejo G, Halberg C, Monschik-Lundin A et al (1998) Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages. *J Lipid Res* 39:755–766
- Miller YI, Shaklai N (1999) Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. *Biochim Biophys Acta* 1454:153–164
- McIntyre JA, Faulk WP (eds) (2008) Special issue: redox and autoimmunity. *Autoimmun Rev* 7:515–584