Autoimmune reactivity of IgM acquired after oxidation

Jasna Omersel¹, Ivica Avberšek-Lužnik², Pegi Ahlin Grabnar³, Tanja Kveder⁴, Blaž Rozman⁴, Borut Božič^{1,4}

¹Chair of Clinical Biochemistry, Faculty of Pharmacy, University in Ljubljana, Ljubljana, Slovenia, ²General Hospital Jesenice, Unit for Laboratory Diagnostics, Jesenice, Slovenia, ³Chair of Pharmaceutical Technology, Faculty of Pharmacy, University in Ljubljana, Ljubljana, Slovenia, ⁴Department of Rheumatology, Division of Internal Medicine, University Medical Centre, Ljubljana, Slovenia

Objectives: Redox-reactive antibodies, mainly of the IgG class, gained a wide area of interest after their autoimmune reactivity was revealed following the application of chemical and physiological oxidants. In this study, we examined the susceptibility of IgMs to oxidation and evaluated their binding to the autoantigens important in some autoimmune diseases.

Methods: IgM and IgG fractions, isolated from healthy individuals' sera, were oxidized using direct electric current or physiological oxidant hemin. Specificities towards beta-2-glycoprotein I (β_2 -GPI), cardiolipin (CL), and rheumatoid factor were evaluated with the enzyme-linked immunosorbent assays (ELISAs). Post-translational modification was investigated by 2,4-dinitrophenylhydrazine reaction.

Results: Electrochemically oxidized IgM fractions exhibited altered immunoreactivity – low to medium titers in anti-CL and low positive titers in anti- β_2 -GPI ELISA but exhibited no rheumatoid factor reactivity. Oxidized IgG and IgM fractions exhibited 2.5- and 5-fold increase in the carbonyl content, respectively.

Discussion: An increase in the carbonyl content along with increased immunoreactivity after oxidation suggests modifications of the IgM paratopes. These results point towards possible modifications of native IgMs to their autoimmune state despite the fact that IgMs were less susceptible to oxidation than IgGs. The importance of an individual's redox status in maintenance of autoimmune reactions was emphasized by *in vitro* diagnostic tests.

Keywords: Autoimmune disease, Protein carbonylation, Natural autoantibodies, Oxidation, Redox-reactive antibodies

Introduction

Many oxidants, as products of *in vivo* reactions or manufacturing processes, were found to react with specific amino acids in the Fc region, resulting in modification of antibody's effector functions.^{1–4} Today, various studies have been published with previously mentioned changes in antigen-binding sites, directly affecting biological activity and specificity, when only one amino acid residue in complementarity determining regions or nearby was affected during oxidation.^{5–7}

A significant increase in reactivity of mono- and polyclonal IgGs isolated from human sera with self-antigens can be detected after modification with a direct flow of electrons,⁸ chemical reagents (HOCl, peroxyxynitrite),⁶ metal catalyzed oxidation (Fe^{2+}),^{8,9}

hemin,^{9,10} neutrophil-driven reactive oxygen species (ROS), and photo-oxidation.⁹ The proposed transformation mechanism, oxidation, resulted in an important alteration in antibodies' immunoreactivity, leading to increased binding to self-antigens.^{11,12}

In autoimmunity, IgMs have often been considered as non-pathogenic, even protective. Binding of IgM to self-antigens blocks their antigenic determinants and prevents autoreactive clones from reacting vigorously with those autoantigens.¹³ Usually, IgGs are the antibodies that mirror the degree of an autoimmune disease, for example in antiphospholipid syndrome, systemic lupus erythematosus, vasculitis, celiac disease, and many others.¹⁴ Among systemic autoimmune disorders, rheumatoid arthritis in particular is a disease in which IgMs are of clinical importance, even though they are also present in many other autoimmune and infectious diseases. IgMs are also an important fraction of so-called natural antibodies

Correspondence to: Borut Božič, Chair for Clinical Biochemistry, Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1000 Ljubljana, Slovenia, EU. Email: borut.bozic@ffa.uni-lj.si

which regulate cellular and enzyme functions by binding as ligands, they participate in the clearance of apoptotic cells, cellular debris, and accumulated oxidized proteins by opsonization, recruit complement, and are probably involved in the regulation of proteolytic activity.^{13,15,16} In addition, they facilitate the functions of the antigen-presenting cells. However, it has been shown that low-affinity natural antibodies, either IgG or IgM type, also react with autoantigens commonly encountered in autoimmune disorders, such as systemic lupus erythematosus and experimental autoimmune encephalitis, and promote a shift from a protective to a detrimental function.¹³ Therefore, autoimmune disorders may simply arise as a consequence of the dysregulation of the physiologically benign autoimmunity. The mechanisms involved in this shift are not completely established. It may be the physicochemical alteration of an autoantigen or its abnormal presentation to autoreactive T-cells.¹³ The latest hypothesis, based on redox reactions, involves previously mentioned alterations in antibody-binding sites and thus altered specificity. These recent findings could be an important contribution to understanding the basic pathology and pathogenetic mechanisms of autoimmune diseases and their multifactorial etiology. Previous oxidation studies were mainly orientated on IgG molecules, analyzing a whole serum or therapeutic intravenous preparation^{9,10} or hybridoma-secreted human or mouse IgG.⁹ There is only one study known to us examining the effect of ferrous ions or heme to polyclonal human IgM with respect to autoimmunity.¹⁷ It has been proven that not all proteins are equally sensitive to oxidative damage.¹⁸ Oxidation susceptibility greatly depends on protein structure (sequence motifs, residues exposed on the molecular surface, additionally bound metal ions), which was demonstrated to also be the case for antibodies.^{7,8}

In this work, as part of our ongoing investigations of redox-reactive antibodies,^{8,12} we have examined the effects of physical and chemical oxidants (electron flow and hemin) on IgM-binding ability with autoantigens. Specific enzyme immunoassays were used to evaluate the immunoreactivity of samples oxidized under a wide range of time and oxidant species concentration. The specific objective of this study was also to analyze one of the possible routes for amino acid modification during oxidation of antibody molecules of both isotypes – IgM and IgG.

Materials and methods

Samples

Serum was separated from freshly harvested blood of seven healthy donors (two men and five women, aged 28–43). Donors were all non-smokers and had no presence of anti-cardiolipin antibodies (aCL),

anti- β_2 -glycoprotein I antibodies (anti- β_2 -GPI), or rheumatoid factor (RF) antibodies determined by specific enzyme-linked immunosorbent assays (ELISAs). Sera were tested as well for the presence of antinuclear antibodies and anti-neutrophil cytoplasmic antibodies by indirect immunofluorescence screening tests where no specific immunofluorescent pattern was observed. Sera were used further for IgG and IgM isolation to obtain material for oxidation experiments. The study was approved by The National Medical Ethics Committee of the Republic of Slovenia and written informed consent was obtained from each subject participating in the study (No.: 163/02/09).

IgM purification

The IgM fraction was purified from human sera by ammonium sulfate cut, followed by size exclusion chromatography. Saturated ammonium sulfate solution was added dropwise to a final 50% concentration. The mixture was stirred vigorously and precipitated overnight (4°C). After centrifugation (30 minutes/2450 × g/4°C), the precipitate was dissolved in phosphate-buffered saline (PBS, 150 mM, pH = 7.4), dialyzed extensively against PBS (150 mM, pH = 7.4), 500 ml at 4°C for 2 hours. Buffer exchange was done in 2-hour intervals, repeated two times, followed by overnight dialysis at 4°C and another 2-hour dialysis in fresh PBS. Finally, the ultrafiltration was performed in stirred cell (Billerica, Millipore, MA, USA) at 4°C to adjust the volume of the dialyzed sample back to its original volume.

The sample was applied at room temperature on a Sephacryl S-300 HR column (1.6 × 100 cm, Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS, pH = 7.4. Proteins were eluted at 10 ml/hour, and collected in ~3 ml fractions. Protein peaks were detected at 280 nm (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer, Camspec Ltd, Cambridge, UK), pooled, and ultracentrifuged. The collected IgM fraction was aliquoted and then stored at –80°C. Purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nephelometry.

For dynamic light scattering analysis, native human IgM protein, 5 mg/ml (AbD Serotec, Kidlington, UK), was further diluted with PBS, pH = 7.4, to obtain a concentration of 0.5 mg/ml, and filtered through 0.22 Sartorius syringe filters (Minisart, Sartorius, Goettingen, Germany).

IgG purification

Purification of IgGs using MAbTrap™ Kit was performed according to the manufacturer's instructions (MAbTrap™ Kit Purification of antibodies, GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Immunoglobulin concentration

Quantitative determination of immunoglobulins in an individual chromatographic peak was made nephelometrically on a BN ProSpec[®] System (Dade Behring, Marburg, Germany) using N Antisera to human immunoglobulins (IgG, IgA and IgM) (Dade Behring, Marburg, Germany) reagent. IgM concentrations used further for the oxidation were between 0.20 and 0.90 g/l. The amount of recovered IgGs was spectrophotometrically determined (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer, Camspec Ltd, Cambridge, UK) using the extinction coefficient of 14.0 for 1% IgG solution.

Oxidation

Electro-oxidation

Separate aliquots (200 µl) of isolated IgM fractions (0.2–0.9 g/l) were exposed to direct current (2, 6.5, and 20 mA), generated with a current/voltage generator (E5263A-E5291A Module, Agilent Technologies) over graphite electrodes (99.999% graphite rods, diameter 3 mm, $l = 150$ mm, SIGMA-ALDRICH) for 15, 30, 60, or 300 seconds.⁶ The electro-oxidation was carried out directly before the ELISA tests.

Hemin oxidation

IgM fractions were separately oxidized with hemin, following the conditions of McIntyre and Faulk.¹⁹ Hemin (Sigma, St Louis, MO, USA) was dissolved in 1.0 M NaOH (15.15 mg/ml). A 200 µl aliquot of isolated polyclonal IgM fraction (0.2–0.9 g/l) was exposed to 5 µl of the hemin solution (with final concentrations of 50, 125 and 200 µM). Following overnight incubation (20–22 hours) at 37°C on a rocking platform, samples were tested in duplicate on a specific ELISA. Five polyclonal IgG fractions were incubated with hemin and used as a hemin activity control test.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was run on 7.5% polyacrylamide gels under non-reducing or reducing conditions, according to Laemmli.²⁰

Size distribution analysis

Size measurements of immunoglobulin monomer and possible polymerization or aggregation after oxidation of IgM and IgG fractions were performed by dynamic light scattering on Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK), which used a 4 mW He–Ne laser operating at a wavelength of 633 nm and a detection angle of 173° (backscatter light detection technology). The following parameters were used:

RI solvent: 1.330; viscosity of solvent: 0.8872 mPa; RI protein: 1.45. All experiments were performed at 25°C in a 1 cm pathlength disposable low-volume cuvette containing 100 µl of the sample. The measurement position and signal attenuation were automatically determined and optimized by the software. A multiple narrow high-resolution mode for fitting the correlogram was used. Results of aggregate populations are primarily shown as sample scattering intensity distribution, the first-order results from a dynamic light scattering (DLS) experiment. The software transformation of data to volume and number distribution was performed with several assumptions: all particles are spherical, have homogeneous and equivalent density, their optical properties are known, and the intensity distribution is without error. Derived calculations presented on graphs are therefore an estimate and are used only for comparison purposes rather than as an absolute measurement.²¹

ELISA tests

Non-oxidized IgM fractions, oxidized IgM fractions, and the original sera were simultaneously tested by the following tests: aCL, anti-β₂-GPI, and RF-IgM ELISA. The sample dilution was modified with a specific dilution factor for each serum sample based on the nephelometrically determined concentration of IgM. To validate the adhesiveness of samples to the plastic surface, stickiness tests were carried out under the same procedure, without coating the microtiter plates with antigens.

To detect aCL activity, an ‘in-house’ solid-phase ELISA (10% fetal bovine serum as a blocking agent), standardized according to the European Forum on Antiphospholipid Antibodies, was performed.^{22,23}

Anti-β₂-GPI activity was determined by evaluated ‘in-house’ ELISA method using purified human β₂-GPI.^{24,25} The level of anti-β₂-GPI was derived from the standard curve according to the defined dilutions of monoclonal antibodies.²⁶

Quantitative testing for IgM RFs was performed by MASTAZYME[™] RF kit (Mast Diagnostica, Reinfeld, Germany) according to the manufacturer’s instructions.

Carbonyl assay for oxidative damage to proteins

The carbonyl group content in protein samples was evaluated by reaction of carbonyl groups with 2,4-dinitrophenyl hydrazine (2,4-DNPH), as described previously with minor modifications.²⁷ A volume of 0.2 ml of the sample (oxidized IgGs or IgMs, sera, and albumin) was allowed to react with 1.0 ml of 2,4-DNPH (10 mM in 2 M HCl) for 90 minutes at 37°C. Protein was then precipitated with 20%

trichloroacetic acid, centrifuged, and washed twice with ethanol/ethyl acetate (1:1). The final protein pellet was dissolved in 6 M guanidine hydrochloride; the concentration of protein dinitrophenylhydrazones was calculated from the maximum absorbance (360 nm) using a molar extinction coefficient of $21.000 \text{ M}^{-1} \text{ cm}^{-1}$. A blank for each sample consisting of non-oxidized immunoglobulin reacted with 2 M HCl, containing no 2,4-DNPH, was carried throughout the procedure. The difference in absorbance between the DNPH-treated samples and the samples treated in parallel with HCl alone was used to calculate the carbonyl content, expressed as nmol of carbonyl per mg protein.

Results

Purity and integrity of IgM fractions before and after oxidation

The stepwise isolation of IgM from human sera gave a pure fraction, without other plasma proteins (Fig. 1). Since IgMs are high-molecular mass proteins they are excluded from the gel and, under reducing conditions, show the expected fragments of molecular mass corresponding to heavy ($\sim 70 \text{ kDa}$) and light chains ($\sim 25 \text{ kDa}$) (lane 4). The absence of other, lower molecular weight bands showed that there was no major IgM cleavage to low molecular weight products after electro-oxidation at 6.5 mA (Fig. 1) or hemin oxidation (data not shown). Still, due to the detection limit of the procedure (approx. $1\text{--}1.0 \mu\text{g}$) we were not able to detect possible dimerized or polymerized products of individually cleaved amino acids, which are expected after radical oxidation of proteins.

Figure 1 SDS gel analysis of non-oxidized and electro-oxidized (6.5 mA) polyclonal human IgM. About $7 \mu\text{g}$ of total proteins were loaded on 7.5% polyacrylamide gel in each lane under non-reducing (lanes 1–3) or in reducing conditions (β -mercaptoethanol; lane 4). Lane 1: non-oxidized IgM; lanes 2 and 3: oxidized IgM 30 and 60 seconds, respectively; lane 4: non-oxidized IgM under reducing conditions. kDa: molecular weight of markers in kiloDaltons. The gel was stained with Coomassie Brilliant Blue.

Oxidation-induced alterations in the specificity of IgM from healthy persons

Electro-oxidized IgM fractions

Aliquots of 7 IgM fractions, isolated from sera of blood donors, were exposed in PBS, pH = 7.4, to direct current-mediated electromotive force over graphite electrodes, using constant current at 2.0, 6.5, and 20 mA for 15, 30, 60, or 300 seconds and $10\text{--}20 \mu\text{A}$ for 5 minutes. Changes in immunoreactivity were measured by specific aCL, anti- β_2 -GPI, and RF-IgM ELISAs.

A maximal and clinically important increase in immunoreactivity was induced with 6.5 mA, after 30 or 60 seconds. The response above the diagnostic limit was equivalent to low to medium positive titers in aCL ELISA and low positive titers in anti- β_2 -GPI ELISA. IgM oxidation using current equal to 2 mA gave negligible immunoreactivity to β_2 -GPI or proteins in aCL ELISA at first, but showed a significant increase after 5 minutes of oxidation; current in the μA range did not result in changes of IgM reactivity to specified antigens. The oxidation of an aliquot with 20 mA resulted in a slight increase above the diagnostic limit of three samples after 15 seconds exposure but a dramatic loss of immunoreactivity after prolonged oxidation (Figs. 2 and 3). Electro-oxidized IgM samples gave no positive values on RF ELISA, irrespective of the current used. The responses of provided controls and our additional control (RF positive IgM fraction) were inside the acceptable range recommended by the manufacturer or were highly positive, respectively.

Hemin-treated IgG and IgM fractions

To verify our hemin-oxidation protocol, we tested 5 IgG fractions isolated from blood of healthy individuals. After overnight incubation with hemin a significant $\alpha\beta_2$ -GPI reactivity of four samples was determined in high or medium titers, with 200 and

Figure 2 Binding of non-oxidized and electro-oxidized healthy blood donors' IgM determined by β_2 -GPI ELISA. Isolated human IgM samples were exposed to electro-oxidation, with a constant current of 2.0, 6.5, or 20.0 mA for 15–300 seconds, and analyzed for altered immunoreactivity against β_2 -GPI. Results are shown as mOD (milli optical density) as a function of current and time of exposure to electro-oxidation.

Figure 3 Binding of non-oxidized and electro-oxidized healthy blood donor IgM determined by aCL ELISA. Isolated human IgM was exposed to electro-oxidation, with a constant current 2.0, 6.5, or 20.0 mA for 15–300 seconds, and analyzed for altered immunoreactivity by aCL ELISA. Results are shown as a mOD (milli optical density) changing with current and time of exposure to electro-oxidation.

125 μ M hemin, respectively. A 50 μ M hemin did not reveal anti- β_2 -GPI reactivity of IgGs. In following experiments with IgM fractions or corresponding sera from healthy individuals, hemin treatment revealed no activity against β_2 -GPI, RF, or proteins in aCL ELISA. Polyacrylamide gel electrophoresis of hemin-treated IgM samples (50, 125, 200 μ M) did not show any fragmentation (data not shown).

Particle aggregation determination

Dynamic light scattering was performed to specify size distribution in immunoglobulins fractions and to assess the aggregation level of IgG and IgM after oxidation. The size distributions of the scattered intensity of the non-oxidized and oxidized IgG and IgM samples are depicted in Fig. 4. The intensity distribution of the IgG sample shows three peaks indicating the existence of three size classes of particles. By intensity, 84% of the distribution contribution is from the smallest particles (14 nm), which represent more than 99.9% of the sample composition by volume. In samples exposed to oxidation for 30 seconds, an additional peak occurred, indicating some aggregation. Although the aggregate intensity peaks at $\approx$ 874 and $\approx$ 146 nm, representing 61 and 33% of the total scattered light, respectively, they represent only 0.1% of the total number of molecules as confirmed by number distribution.

In IgM samples, the distribution shows three peaks, again indicating the existence of three size classes of particles. By intensity, 21% of the distribution contribution is from the smallest particles (17 nm), representing 96% of the sample composition by volume. In samples oxidized for 30 seconds, aggregation of molecules increased as in the case of IgG. Although the aggregate intensity peaks at $\approx$ 375 and $\approx$ 87 nm, representing 59% and 34% of the total scattered light, respectively, they represent 32% and 4% of the total volume of molecules, respectively. The percentage of IgM aggregation increased with the time of

exposure to oxidation. After 60 seconds, the 17 nm population disappeared and the largest aggregates at $\approx$ 375 and $\approx$ 87 nm increased to 34 and 66% in volume distribution.

Carbonyl group assay of oxidatively modified fractions of antibodies

Carbonyl content, a marker of protein oxidation, was measured in non-oxidized and oxidized IgG and IgM fractions to examine the effect of electro-oxidation on antibody amino acid composition. The carbonyl content of non-oxidized and oxidized bovine serum albumin (BSA) was measured as an assay control, with the response being linear with increasing protein concentration. In all tested sera, protein carbonyl content was less than 1 nmol/mg protein (Fig. 5A).

Protein carbonyl content increased approximately 2.5- or 5-fold in both oxidized IgG and IgM fractions as compared to corresponding non-oxidized controls. However, the levels of carbonyl content after 30 and 60 seconds did not differ significantly (Fig. 5A).

Discussion

Since 2004, a novel family of antibodies, capable of recognizing autoantigens subsequent to oxidation-reduction reactions, has been studied intensively. Such redox-reactive antibodies, circulating in blood and in breast milk of humans and animals, proved to play a beneficial role in some physiological processes: the removal of senescent red blood cells, malignant or apoptotic cells, the clearance of oxLDL via Fc receptors, etc.²⁸ However, it is suggested that part of these redox-reactive antibodies can acquire autoimmune properties as well after the redox reaction, most likely on amino acids in their antigen-binding site. The microenvironment providing such conditions can be established locally at the site of intensive inflammation (oxidative burst by phagocytic leukocytes) or as a consequence of decreased function of enzymatic and non-enzymatic antioxidant systems, e.g. normal aging and disease, all of which was also found in patients with rheumatoid arthritis and juvenile arthritis.^{29–31} Free radicals themselves or their by-products (malonaldehyde, 4-hydroxynonenal, etc.) are altogether leading to the harmful condition called oxidative stress, promoting oxidation of antibodies.

Previous studies confirmed oxidation of immunoglobulins by oxidants or variations in the medium redox potential. Primarily experiments were done on the most common isotypes, IgG; results shown herein broaden the understanding of oxidation susceptibility of IgM. Although both isotypes exhibited some aggregation after oxidation, specific analysis of monomer/oligomer confirmed alterations in

Figure 4 Intensity size distributions for IgG and IgM samples: (A) non-oxidized samples; (B) samples oxidized 30 seconds (6.5 mA); (C) samples oxidized 60 seconds (6.5 mA). The distribution, obtained from a DLS measurement, is based on intensity of scattering which is proportional to R^6 (R = radius), from Rayleighs approximation. The area under each peak is proportional to the relative amount of scattered light of each particle family. Mie theory is then used to convert intensity distribution to a volume distribution (on picture: % of intensity and % of volume) which shows the importance of the each single peak in oxidized fractions.²¹

immunoreactivity towards acquired autoimmune specificity, being less significant in the case of IgM.

Immunoreactivity and immunoglobulin aggregation after oxidation

ELISA tests of oxidized IgMs clearly showed dynamic changes in immunoreactivity to previously specified antigens. Stickiness tests of samples ruled out non-specific adsorption of oxidized antibodies on a plastic microtiter plate surface. Compared to oxidized IgGs, no detectable immunoreactivity changes were observed with IgMs using 1.5 V (μ A range), which indicated a need for higher concentration of oxidant species for a similar biological response due to the IgMs' complex pentameric structure. Indeed, maximal and clinically important increases in the immunoreactivity of oxidized IgMs in both anti- β_2 -GPI and aCL ELISAs were then induced after 30–60 seconds with 6.5 mA (the current used previously to achieve maximal response on an isolated IgG fraction), although the alterations in IgGs were mostly detected earlier, after 15 seconds and even with lower current.¹² As expected, prolonged oxidation at 6.5

and 20 mA led to decreased immunoreactivity in all cases, determined for both isotypes.¹²

DLS analysis, a sensitive and non-invasive technique for measuring particle size, was performed to follow a possible aggregation of immunoglobulins during oxidation. The intensity size distributions of non-oxidized IgG and IgM (Fig. 4A) indicates the existence of three size classes of particles where the area under each peak is proportional to the relative amount of scattered light of each particle family. The population with the highest diameter is probably the consequence of spontaneous aggregation of IgG and IgM. By scattered light intensity, 18% of the distribution contribution of IgM is from the largest particles (8% of IgG), which by volume represent only 0.5% of the size distribution (0.1% of IgG). Results confirmed the expected instability and aggregation possibility of native immunoglobulins in a PBS buffer without stabilizers and after isolation, especially in the case of IgMs, which are more subject to spontaneous aggregation.

Volume distribution of 30 seconds oxidized fractions of IgG and IgM shows that the amount of

Figure 5 Determination of protein carbonyl content. (A) BSA (assay control), serum, and antibody fraction samples (non-oxidized, oxidized) were derivatized with 2,4-DNPH. Carbonyl content was determined spectrophotometrically at 370 nm. Values are expressed as mean $\pm$ SD; $N = 5$ samples per group, and expressed as nmol carbonyl per mg protein. (B) A linear relationship between albumin carbonyl content and its concentration is shown as an assay control.

aggregates larger than 200 nm is ≈ 50 and 32% for IgG and IgM, respectively. However, results on $\alpha\beta_2$ -GPI ELISA, measuring the residual immunoreactivity after filtration of aggregates larger than 200 nm, confirmed about 70% retained activity of the residual monomer (oligomer) immunoglobulin fraction. In IgG samples, the aggregation did not increase if the samples were exposed to oxidation for 60 seconds. On the contrary, the percentage of IgM aggregation increased with time of exposure to oxidation. In samples oxidized for 30 seconds, the smallest population, IgM monomer/oligomer, was still present (64% volume, 68% residual immunoreactivity) but disappeared in fraction oxidized for 60 seconds where 62 nm-sized population increased in its volume distribution and lost its immunoreactivity towards β_2 -GPI in ELISA.

Electro-oxidation – mimicking in vivo oxidation and leading to carbonylation

The magnitude and the post-translational form of proteins greatly depend on amino acid moieties and an oxidizing agent. First, the electrochemical modification, a common method for amino acid modification and detection^{32–34} or protein oxidation

cleavage,^{35,36} was used in our studies to mimic *in vivo* oxidation; its final consequences can be compared to the *in vitro* system of stimulated neutrophil cells. Two simultaneous process are carried out in the oxidation cell: electron-transfer for direct protein oxidation and indirect oxidation with powerful oxidizing agents, e.g. hypochlorite, or even ROS, formed during controlled medium (PBS) electrolysis. The only previously reported modification of such redox-reactive antibodies was the nitration of aromatic amino acids in an autoantibody's hypervariable binding site,¹¹ which is very likely to be a result of specific experimental conditions. In our study, oxidized immunoglobulin fractions, derivatized with 2,4-DNPH, demonstrated significant increase in carbonyl content compared to non-oxidized fractions. This constitutes a novel transformation possibility, implicating variations in amino acid side chains. The unusually high values of carbonyl content in oxidized immunoglobulins reflect more aggressive oxidation conditions than those during oxidative stress. Such specific modification of proteins has previously been observed as well as a consequence of neutrophil activation and the production of a strong oxidant, hypochlorous acid, after increased myeloperoxidase activity.³⁷ Increased carbonyl content in the gamma-globulin fraction of patients with juvenile idiopathic arthritis was reported to be a potential marker of inflammatory process activity.³⁸ Considering our results this covalent modification of antibodies influences their spatial form and increases the flexibility of molecules (especially in complementarity determining regions), reflected in increased immunoreactivity and alteration of IgM's and IgG's specificity.

Under our experimental condition, IgG fractions incubated with a 50 μ M hemin solution did not bind to β_2 -GPI and CL in a clinically important manner; however, the active oxidizing effect of hemin on IgG fractions was proved with 125 and 200 μ M hemin on anti- β_2 -GPI ELISA which is also in agreement with McIntyre and Faulk, where immunoreactivity of IgGs was revealed in aCL ELISA.¹⁹ In testing hemin-oxidized IgM, ELISA tests for anti- β_2 -GPI, aCL, and RF antibodies failed to demonstrate auto-reactivity, irrespective of the hemin concentration used. A moderate increase in RF activity has been previously observed after the exposure of therapeutic polyclonal intravenous immunoglobulin to hematin but resulted in no further antigen-binding activity after treatment with ferrous (Fe^{2+}) ions.³⁹ Based on our previous experience, there are sera which lack redox-reactive antibodies under previously used conditions indicating intra and interindividual variability: Not all IgGs in polyclonal fraction are equally sensitive to oxidation⁴⁰ and IgM's susceptibility to oxidation, resulting in autoimmune activity, is even

more restricted. Heme and hemin are known to induce toxicity through the generation of ROS and proinflammatory effects.⁴¹ Thus negative results of hemin-oxidized IgMs could point toward their protective role. When detoxification systems get overwhelmed, this results in protein oxidation, aggregation, or degradation causing severe cell or tissue damage and diverse pathology conditions.⁴¹

In conclusion, IgMs were found to be less susceptible to oxidatively induced alterations than IgGs, which we assume is a consequence of their complex structure as well as of their different role in immunological responses. The residual immunoreactivity, obtained on anti- β_2 -GPI ELISA tests after filtration of aggregates in oxidized samples, confirmed our hypothesis that oxidized monomers or oligomers also possess autoimmune reactivity. In addition, oxidation with hemin and electric current confirmed that it is not only the amino acid sequence/protein structure but also the nature of the oxidation process itself which contributes to diverse effects on antigen binding. Along with the clinical studies of McIntyre and co-workers^{19,42} and kinetic and thermodynamic analyses of Dimitrov *et al.*,⁴³ our results support the hypothesis of immunoglobulins' redox reactions being involved in autoimmune mechanisms.

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