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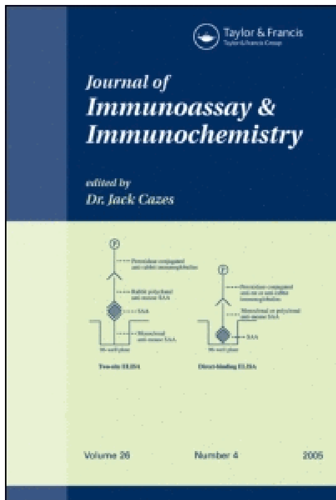

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ALTERATION OF ANTIBODY SPECIFICITY DURING ISOLATION AND STORAGE

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ALTERATION OF ANTIBODY SPECIFICITY DURING ISOLATION AND STORAGE

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□ *Isolation buffer characteristics and storage conditions could partially transform natural antibodies. 50 IgG factions were isolated from seven healthy donors' sera using various protein G columns and buffers. PAGE revealed no major antibody cleavages; purity of IgGs eluted at pH 2.4–3.5 was close to homogeneity, independent of buffer composition. Although eluting at pH 2.4 resulted in 3.5- or 17-fold higher antibody yield compared to pH 3.0 or 3.5, respectively, it distorted the antibody molecule. IgGs eluted at pH 2.4 acquired reactivity against diagnostically important autoantigens, confirmed by standardized ELISAs. Preserved antibodies' natural activity is important in further experiments with oxidatively-induced autoantibodies.*

Keywords acidic elution buffer, antibody purification, autoimmunity, immunoreactivity, redox-reactive antibodies, specificity

INTRODUCTION

On the very basic structural level, amino acids comprising antibodies are arranged in two identical light and two identical heavy chains linked together by covalent disulphide bonds, non-covalent bonds and dominant hydrophobic interactions. Amino acid composition is generally conserved among classes of antibodies, but still unique in antigen-combining sites. Besides flexibility in the hinge region between CH1 and CH2 domains, this accounts for the capacity of different antibodies to bind an immense number of diverse antigens. This repertoire can be enlarged considerably when amino acids are subjected to one of the common transformation processes. In addition to total chemical or physical degradation, a partial region specific transformation of

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protein/antibody is often dependent on pH, buffer components and concentration, presence of excipients (e.g., in protein drug formulations), and storage conditions.[1] All these factors should be considered from the start – from synthesis, purification, identification – to the final use of a protein sample.

Lately, there has been special interest in investigations of so-called redox-reactive or "masked" antibodies, which now have been accepted as a special or unique subset of natural autoantibodies, ubiquitous in normal human sera.[2] These antibodies are more liable to oxidation changes, irrespective of their original specificity. It has been reported that their avidity or specificity can be altered in an oxidation process. During the first stage, electron-rich side groups of amino acids in hypervariable regions are affected, followed by extensive oxidation in the other parts of molecule. The latter probably leads to a major conformational disorder and, finally, to antibody denaturation.

Besides the common theory that oxidation can convert some antigens to autoantigens, this is a novel theory for the springing up of an autoimmune disease. It was experimentally shown that oxidatively transformed natural antibodies acquire binding capacity towards various autoantigens, important in autoimmune disease, like cardiolipine (CL), beta-2-glycoprotein I (β_2-GPI), lupus anticoagulant, cyclic citrullinated peptide (CCP), and nuclear and cytoplasmic antigens.[3,4] Oxidatively induced autoimmune antibodies have not been fully described yet; their stability and activity characteristics as well as a needed oxidizing agent and the types of chemical transformations in a molecule are not totally understood. Bearing this in mind, we became aware of the importance of the proper conditions for IgG isolation, especially when further experiments are planned on electro-oxidation – the method which helped us reveal the antigen binding properties of redox-reactive autoantibodies.

There are many well-known purification procedures of antibodies, ranging from very crude (precipitation of sample proteins in the range of Igs with ammonium sulphate, caprylic acid or polyethylene glycol) to more specific, which involve the binding of antibodies to covalently immobilized ligands (protein A, G, L).[5] Each method is based on different procedures and exploits advantages of various physical properties of the proteins being isolated.

In this report, we compare the yield, the integrity, and the purity of IgG fractions, isolated with affinity chromatography under different elution conditions. Modifying the elution conditions could help us choose the best procedure for isolating structurally unaffected IgG fractions with a satisfying yield, suitable for further investigation of basic characteristics of very unstable redox-reactive antibody fractions.

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EXPERIMENTAL

Serum Samples

Serum samples from seven healthy blood donors were selected. ANA tests by indirect immunofluorescence on HEp-2 cells, antibodies on extractable nuclear antigens (ENA) by counterimmuno-electrophoresis (CIE) and anti-β_2-glycoprotein I (anti-β_2-GPI) by enzyme-linked immunosorbent assay (ELISA) were negative. Two of sera exhibited low positive titers of anti-cardiolipin antibodies (aCL) by ELISA, others were negative. All sera were fresh and non-decomplemented.

Immunoglobulin Class G Isolation

50 polyclonal IgG fractions were isolated by affinity chromatography out of seven human sera under different elution conditions. The isolation of IgGs was performed using two commercially available kits with the original and modified buffers:

a) ImmunoPure® (G) IgG Purification Kit (Pierce Biotechnology, Rockford, IL). Serum samples were diluted with an equal volume of ImmunoPure® (G) Binding Buffer, filtered through a 45 µm filter (Minisart®, Sartorius, Goettingen, Germany) and applied onto a column. By adding an adequate volume of the Binding Buffer, all unbound proteins were thoroughly washed away, which was verified by the last flow-through absorbance (280 nm) being equal to the Binding Buffer alone. Bound IgGs were eluted from the column using IgG Elution Buffer (pH 2.8) and immediately adjusted to the physiological pH by adding 1 M Tris. Fractions containing a significant amount of the protein were pooled and desalted to PBS (pH 7.4) using a desalting column.In additional experiments, IgGs were eluted from the columns at pH 2.4, pH 3.0 or pH 3.5 by the same procedures as described above, with two exceptions: 0.7 M acetic buffer (pH 5.2) was used for sera dilutions and washing away the unbound proteins; the original elution buffer was substituted with 1 M, 58 mM, 5.8 mM acetic acid with pH 2.4, pH 3.0 or pH 3.5, respectively. After elution with pH 3.0 or pH 3.5, some IgGs remained bound to the column, and were subsequently eluted with 1 M acetic acid, neutralized, desalted and stored at −80°C.
b) MabTrapTM Kit (Amersham, GE Healthcare, Little Chalfont, UK). Buckinghamshire Purification of IgGs using MabTrapTM Kit was performed according to the manufacturer's instructions. Briefly, the sera were diluted 1:1 with the Binding Buffer, filtered (45 µm, Minisart®, Sartorius, UK) and passed through the column. After

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washing away the unbound serum components, the retained IgGs were eluted (pH 2.7) and 1 mL fractions collected in tubes containing Neutralizing Buffer. Fractions were desalted to PBS (pH 7.4) using a desalting column (ImmunoPure®(G)).

Total Protein Estimation

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.

Time Dependent Influence of Low pH on Serum Specificity

To determine the effect of pH on the natural antibodies' specificity, serum solutions at pH 2.4, 3.3 and 5.2 were analysed. One serum (serum D) was diluted with acetic acid (Sigma-Aldrich, Seelze, Germany) (serum vs. acid – 1:4.4), 1 M acetic buffer (pH = 2.4), 0.7 M acetic buffer (pH = 5.2) and 0.85 M acetic buffer (pH = 3.3). In order to assess the influence of the exposure time of the serum D to an acidic pH environment on antibodies' specificity, the neutralization with 1 M Tris (Merck, Darmstadt, Germany) was performed after 1, 2, 5, 10, and 15 min.

To evaluate the influence of the serum vs. acid ratio on antibodies' specificity, a similar experiment with 1 M acetic acid (ratio 1:8) was performed as described above and neutralized after 1, 5, and 15 min.

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Analysis of Autoantibody Specificity

Non-treated sera, acid treated sera, and isolated IgG fractions eluted at different pHs were simultaneously tested by the following tests: aCL ELISA, anti-β_2-GPI ELISA, and anti-cyclic citrullinated peptide (aCCP) ELISA. The IgG concentration in isolated fractions and acid treated sera were individually adjusted to a 100-fold dilution of the non-treated serum as suggested in ELISA protocols.

aCL ELISA

An "in house" solid phase ELISA, standardized according to the European Forum on Antiphospholipid Antibodies, was used to detect aCL activity.[6,7] Briefly, polystyrene microtitre plates (Medium binding, Costar, Cambridge, Massachusetts, USA) were coated with cardiolipin

(Sigma, St. Louis, USA) and blocked with 10% foetal bovine serum (FBS) (Sigma, St. Louis, USA) in phosphate-buffered saline (PBS), pH 7.2. After washing with PBS (pH 7.2), appropriately diluted samples in 10% FBS-PBS were applied and incubated for 2.5 hours at room temperature (RT). Afterwards, plates were washed and incubated with alkaline phosphatase conjugated goat anti-human IgG (ACSC, Westbury, USA) at RT for one hour. After four washings, 100 μL/well of p-nitrophenyl phosphate (Sigma, St. Louis, USA) in diethanolamine buffer (pH 9.8) was added. Developed plates were analyzed spectrophotometrically at 405 nm by a microtiter plate reader (Tecan, Grödig, Austria).

Anti-β_2-GPI ELISA

Anti-β_2-GPI activity was determined by an "in house" method[6,8] evaluated through the European Forum on Antiphospholipid Antibodies.[9] In brief: polystyrene microtitre plates (High binding, Costar, Cambridge, Massachusetts, USA) coated with 50 μL/well of β_2-GPI (10 mg/L) in PBS (pH 7.4), were incubated for two hours at RT. The plates were then washed with PBS (pH 7.4) containing 0.05% Tween (PBS-Tween) and incubated with samples diluted in PBS-Tween for 30 minutes at RT. The detection system was the same as in aCL ELISA. The level of anti-β_2-GPI was derived from the standard curve according to the defined dilutions of monoclonal antibodies.[10]

aCCP ELISA

Testing for IgG aCCP was performed by an Immunoscan CCPlus kit (Euro-Diagnostica AB, Malmö, Sweden) according to the manufacturer's protocol.

Electrophoresis

The purity and integrity of desalted IgG eluates were assessed according to Laemmli by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), on the Mini-Protean II apparatus (Bio-Rad, Hercules, CA, USA). The procedure was carried out under non-reducing or reducing conditions, using 4% stacking and 7.5% resolving gel.[11] For each IgG fraction, volumes containing 5 or 10 μg of total proteins were adjusted to 22.5 μL with PBS (pH 7.4) and mixed with 7.5 μL of sample buffer. Proteins were run for 60 min at 125 V, stained with a Commassie brilliant blue R solution (Sigma, St. Louis, USA) and scanned with G-box (Syngene, Cambridge, United Kingdom).

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RESULTS

Preserved integrity of antibodies, the yield and the purity of isolated IgG were considered during the isolation process.

Purification of Immunoglobulins from Crude Samples

Protein G columns (Pierce and Amersham) with different elution buffers were evaluated for their capability of enriching class G immunoglobulins from crude human sera and to evaluate the level and nature of possible contaminants in isolated IgGs.

IgG fractions, isolated with elution buffers of unknown and known composition, and different pHs, were loaded onto a SDS-PAGE, 7.5% polyacrylamide gel under either non-reducing or reducing conditions.

As displayed in Figure 1, all samples showed high and comparable antibody purity. A major group of multiple bands seen from lanes 1–4 represents a fraction of polyclonal, monomeric IgGs. The last was confirmed by running the sample in lane 2 under reducing conditions with 0.5% β-mercaptoethanol (lane 5). Two bands in lane 5 represent heavy and light chains of IgG at ~50 kDa and ~25 kDa, respectively. Only minor impurities of low molecular weight (lanes 1, 2 and 3), as well as some high

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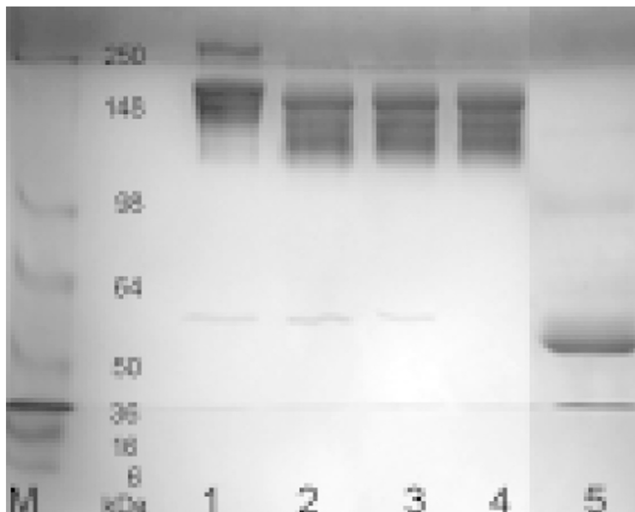

FIGURE 1 SDS-PAGE of polyclonal IgG purified with protein G affinity chromatography and eluted under different conditions. About 5 μg of total proteins were loaded on 7.5% polyacrylamide gel in each lane in non-reduced (lanes 1, 2, 3, 4) or reduced conditions (β-mercaptoethanol; lane 5). Lane 1: IgGs eluted at pH 2.4; lane 2: IgGs eluted at pH 2.7; lane 3: IgGs eluted at 2.8; lane 4: IgGs eluted at

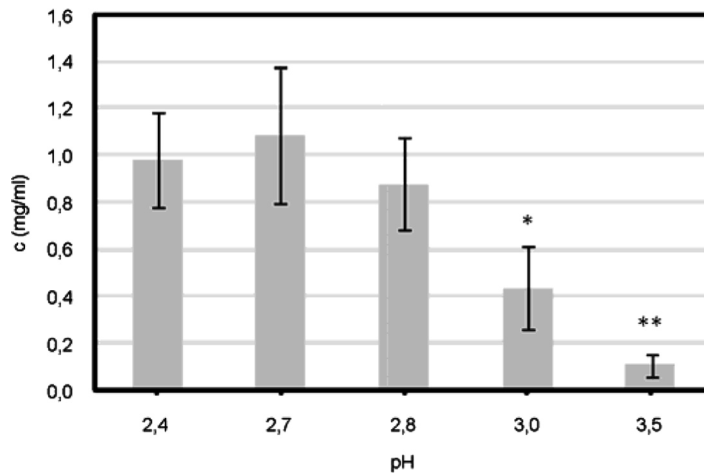

FIGURE 2 The influence of elution pH on the amount of recovered IgGs. x-axis: affinity elution at pH 2.4, 2.7, 2.8, 3.0, and 3.5; y-axis: average concentration ± SD of recovered IgGs (mg/mL) at specific pH; * recovery of IgG eluted at pH 3.0 compared to pH below 3.0 ($p < 0.05$, t-test); ** recovery of IgG eluted at pH 3.5 compared to pH below 3.0 ($p < 0.05$, t-test).

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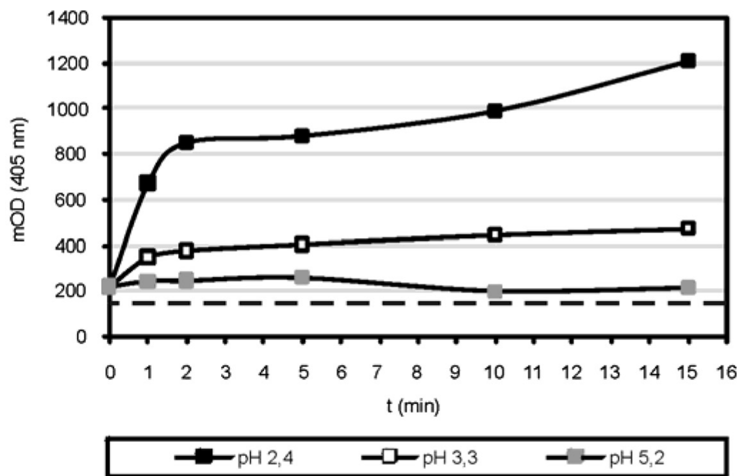
molecular weight over 250 kDa (lane 1) were seen. The latter was a specific observation in 6/7 isolated fractions at pH 2.4 only.

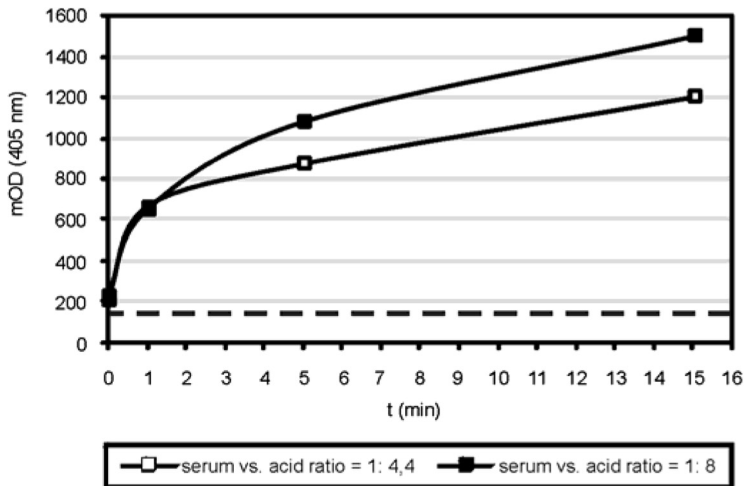
The overall concentrations of IgG fractions, recovered under different conditions of isolation, were calculated from A_{280nm} values (Figure 2). Elution at pH 2.7 with glycine-HCl (MAbTrap Kit) yielded the highest, although not statistically different ($p > 0.05$, t-test) amount of IgGs compared to the ones at pH 2.4 (1 M acetic acid) and at pH 2.8 (Immunopure (G) IgG Purification Kit). The amount of recovered IgGs diminished significantly at pH 3.0 and 3.5 ($p < 0.05$, t-test). Moreover, there was a 7- to 23-fold reduction in recovery at pH 3.5, and 2 to 3.8-fold at pH 3.0 compared to that at pH 2.7.

Time Dependent Influence of Low pH on Serum Specificity

To determine the effect of pH on IgG specificity, one serum (serum D) was treated at different pH (2.4, 3.3, and 5.2) and neutralized after 1, 2, 5, 10, and 15 min. Non-treated serum and neutralized serum solutions were simultaneously tested on aCL and anti-β_2-GPI ELISAs. At pH 2.4 the level of aCL immunoreactivity increased from a low positive level (non-treated serum) to a medium positive level after one minute of incubation and to a high positive level at longer incubation periods. At pH 3.3, an increase to a medium positive titer was observed, whereas at pH 5.2, no enhancement in aCL signal was detected (Figure 3a). Also, no meaningful elevations in anti-β_2-GPI titers in any of the experiments were detected.



(a)



(b)

FIGURE 3 (a) Time dependent effect of pH on the immunoreactivity in aCL ELISA. x-axis: time at 0, 1, 2, 5, 10, 15 min. y-axis: mean absorbance value (mOD_{405nm}). Diagnostically important absorbance limit, calculated from standard curves, is fixed in all figures with dashed line. (b) Time dependent effect of the ratio serum vs. acid on the immunoreactivity in aCL ELISA. x-axis: time at 0, 1, 5, 15 min. y-axis: mean absorbance value (mOD_{405nm}). Diagnostically important absorbance limit, calculated from standard, is fixed in all figures with dashed line.

To address whether the ratio of serum vs. acid affects the degree of a titer, aCL signals of two solutions (1:4.4 and 1:8) were compared. The results showed that aCL titers of both solutions increased from low positivity (non-treated serum) to medium positivity after 1 min and to high

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positivity after 5 or 15 min of incubation. However, titer values after 5 and 15 min were approximately 25% higher at the 1:8 ratio (Figure 3b).

Effect of pH and Elution Buffer Composition on Changes in Immunoreactivity

Fresh human sera from seven blood donors were collected and tested on aCL, β_2-GPI and aCCP ELISAs. Five samples (A, B, C, E, F) were determined to be negative on all tests, two (D, G) were determined as low positive in aCL ELISA (Figure 4).

Isolated IgG fractions, eluted with buffers of different composition and pH, were analysed on ELISAs for changes in their immunoreactivity against aCL, aβ_2-GPI, and aCCP and compared to their respective non-treated sera.

Binding of IgGs with CCP (Figure 5) with β_2-GPI (data not shown) or with aCL (Figure 4) in relevant ELISAs depended strongly on the pH used during the IgG isolation process. In aCL ELISA, all seven samples showed binding in the diagnostically important range with the antigens when pH 2.4 was used for elution from the affinity column. pH 2.7 or above did not change the immunoreactivity of eluted IgG in aCL ELISA. The same phenomenon was observed in aCCP ELISA where at pH 2.4 eluted IgG fractions became reactive with CCP. An increase from 5 to 10 international units (IU), compared to relevant untreated sera, was measured in 6/7 samples.

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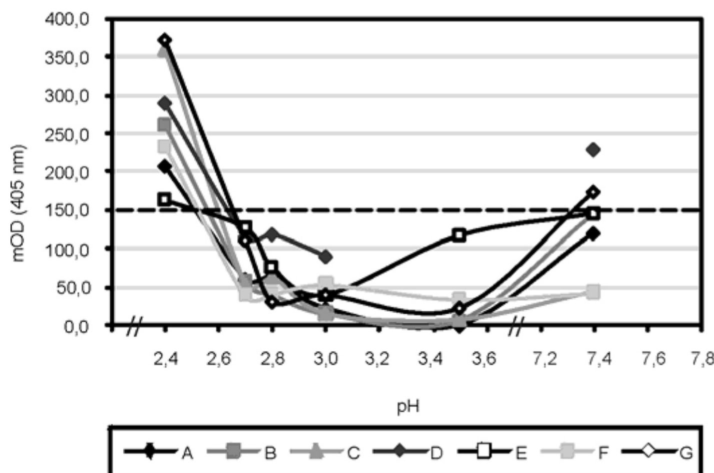

FIGURE 4 Effect of the elution buffer's pH on changes in immunoreactivity of IgG fractions in aCL ELISA compared to their original sera. x-axis: affinity elution at pH 2.4, 2.7, 2.8, 3.0, 3.5 and sera at pH 7.4. y-axis: mean absorbance value (mOD $_{405nm}$). Dashed line indicates a limit above which samples are considered positive.

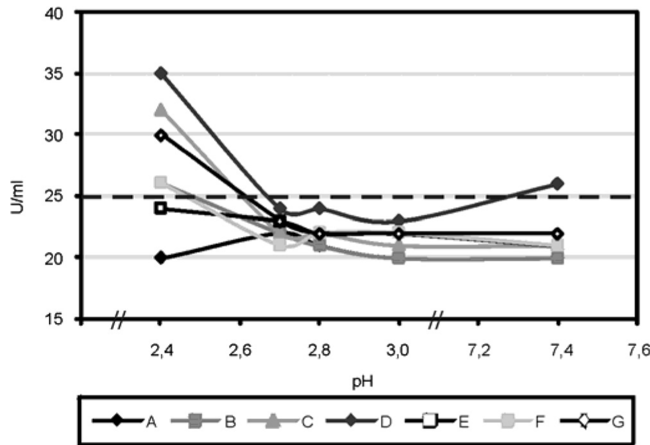

FIGURE 5 Effect of the elution buffer's pH on changes in immunoreactivity of IgG fractions in aCCP ELISA compared to their original sera. x-axis: affinity elution at pH 2.4, 2.7, 2.8, 3.0, 3.5 and sera at pH 7.4. y-axis: Dashed line indicates value of 25 U/mL – a limit above which samples are considered positive.

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No binding of fractions isolated with the original buffers of either manufacturer, which differ greatly in ionic compositions, was observed either in aCL or in aCCP ELISA.

Thermo-Stability of Oxidized Antibodies

Ten samples of IgG isolated from ten blood donors, exhibiting a high immunoreactivity to β_2-GPI tested on anti-β_2-GPI ELISA after electro-oxidation

TABLE 1 The Influence of Temperature on the Thermo-Stability of the Oxidized IgGs – Expressed as the IgG Binding Ability to β_2-GPI After Storage

Sample	Time [h]	T	AUG*	% Conserved Binding to β2-GPI
X	0	20°C	92,0	100
	265	−80°C	84.7	92
	265	4°C	33.8	37
	40	37°C	79.75	87
Y	0	20°C	65.2	100
	265	−80°C	77.7	119
	265	4°C	26,4	40
	40	37°C	80.3	123
W	0	20°C	41.1	100
	265	−80°C	69.7	169
	265	4°C	32.1	78
	40	37°C	46.9	114

*Arbitrary units for IgG.

with 4.5 V, were re-analyzed after storage for one-year at −80°C. A comparison to "freshly" oxidized samples showed that in 5/10 samples immunoreactivity diminished by about 50%, in 2 samples even by 70%. Separately, the influence of temperature on thermo-stability of oxidized IgGs was studied on three different randomly chosen blood donors' IgG fractions (marked as X, Y, W in Table 1). Aliquots of the same sample were oxidized for 60 seconds at the potential 4.5 V (direct current)[4] and stored at different temperatures (−80°C, +4°C, +37°C). The highest reversibility of oxidative alterations was observed at +4°C (Table 1).

DISCUSSION

Purification of immunoglobulins is generally a compromise between yield, purity, and preservation of their initial, natural immunoreactivity. It should be taken into consideration that any method that includes harsh chemical, physical, or enzymatic treatments during the isolation process can directly affect antibodies' integrity, their binding properties and also, their (immuno)reactivity.

Although a brief exposure of an antibody to a usually acidic elution buffer (range of 2.0–3.0), followed by an immediate neutralization of eluate does not inactivate or even degrade a protein molecule, this seems to be one of the most crucial factors in the IgGs isolation procedure. Not all, but some antibodies are extremely unstable and may irreversibly denature or reorganize their native state[12] and, thus, change their binding properties under acidic conditions, both at 20°C and by even more at elevated temperatures.[13,14] Indeed, some of our previous experiments revealed unexpected binding of IgG fractions isolated from blood donors to autoantigens like β_2-GPI, CL on ELISA tests (our unpublished data). Such unexpected bindings have already been observed on previous reported studies, but it appears that each specific antibody, possessing a unique amino-acid composition in antigen binding regions should be re-tested after isolation, for its remaining biological activity. Usami et al. reported the decreased neutralizing capacity of a monoclonal antibody (C23) – a possible passive immunotherapeutic agent against cytomegalovirus – after 14 days storage in an acidic, basic, or oxidative environment.[15] The importance of a suitable purification method for immunoglobulins was discussed by Bergmann-Leitner et al. with a concluding remark that the efficiency of a purification method differs on the species of IgG donors; the purification itself did not affect the avidity of MSP-I 42 specific antibody but influenced its biological activity depending on the method used.[16]

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Yield, purity, and immunoreactivity changes of polyclonal IgGs against some common autoantigens (β_2-GPI, protein-CL complexes, CCP) were evaluated after the elution of antibodies from columns with different pHs and/or different elution buffers. Comparing the yield of purification, the highest rates were achieved with glycine-HCl pH 2.7, followed by Immunopure (G) IgG Purification, pH 2.8, and acetic acid, pH 2.4, which also gave significantly higher yields than elution at and above pH 3.0. The latter is probably due to the low chaotropic strength of acetic acid at pH 3.0 and its consequent insufficient disruption of charged and polar interactions between protein G and the antibody's Fc region.[17,18] On the other hand, high recoveries at pH 2.7 and 2.8 indicate that not only low pH, but also elution buffer composition (e.g., a possible presence of chaotropic salts) affects the recovery. Considering only the yield, our results confirmed that all three elution buffers with pH below 3.0 gave enough starting material for further analyses of electro-oxidative alteration of antibodies' specificity.

In *in vitro* electro-oxidation experiments, we have been streaming towards maximum control over the conditions and minimum complexity and, thus, the highest purity, of the system. Therefore, the purity and the integrity of the isolated fractions are imperative and were also assessed by SDS–PAGE. The results confirmed there were no major degradation products present, regardless of low pH or buffer composition. Low intensity bands at ~50 kDa (lanes 1, 2, 3) are most likely the result of partial degradation of antibodies, due to acidic elution conditions. The quantity of degradation products is negligible compared to the isolated fractions as a whole.

Based on our previous experiments, it was obvious that the cause of specificity alteration lies in the purification procedure. In order to confirm our suspicion that the acidity of the elution buffer is liable for alteration in the specificity of antibodies and to better define the critical point during the elution, a serum was mixed with the same solutions to which it was exposed during the elution and was tested on aCL and on anti-β_2-GPI reactivity. The exposure of the serum to the elution buffer alone (pH 2.4) significantly affected the specificity of antibodies and resulted in medium, clinically significant titers/levels on aCL ELISA, which are usually associated with the clinical manifestations of antiphospholipide syndrome.[19] The observed phenomena increased with the time of serum exposure to an acidic environment and, by increasing the acid vs. serum ratio (toward greater acidity). This observation is also consistent with our opinion that low pH induced alterations in the specificity of antibodies are due to partial site-specific oxidation rather than to dissociation of natural inhibitors in a serum.[20]

Our results demonstrated that there was no antibody cleavage, but alterations in immunoreactivity after IgGs' elutions at low pH, especially at pH 2.4 (1 M acetic acid). In all seven samples eluted at pH 2.4, diagnostically important

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elevations in the immunoreactivity of autoantibodies were determined with relevant ELISA tests, with the highest response for aCL. This was not a surprise because target antigens in aCL ELISA are several plasma proteins bound to cardiolipin, thus exposing a variety of binding sites for antibodies.[21] Important elevations in the quantities of antibodies against CCP and β_2-GPI were observed. They serve as diagnostic markers for rheumatoid arthritis and antiphospholipide syndrome, respectively.[22–24]

In a study from Narhi et al., the effect of three types of elution buffers, with a distinct chaotropic and denaturing capability on recovery and structure of monoclonal antibodies after affinity chromatography, was tested.[18] Compared to urea (7 M, pH 4.0) and guanidine hydrochloride (6 M, pH 4.0), both of which caused disordered a secondary antibody structure with less β-sheet structures and partial unfolding, antibodies in 0.1 M glycine (pH 2.9) have undergone only small extent of denaturation. Antibodies subsequently refolded in PBS (or urea/PBS) still exhibited the ability to form stable complexes with native antigens, but most probably by recognizing different epitopes.[18]

Thermodynamic analyses showed that oxidative alterations of native and antimicrobial antibodies to autoantibodies are reversible. Incubation time at each temperature (−80°C, +4°C, +37°C) was calculated on the basis of Q_{10} temperature coefficient, often being ~2 for biological systems when assuming that the rate of reaction increases by two times when the temperature is raised by ten degrees. Electro-oxidized samples stored at +4°C lost their binding potential to β_2-GPI by more than 60%, which is still in agreement with data already reported about the remaining activity after a prolonged period of time.[3,25] Following one-year of storage at −80°C, oxidized IgGs exhibited significantly (up to 70%) diminished binding to β_2-GPI, in spite of low temperature. Additionally, our experiments point to inter-individual differences among tested sera, embracing a diverse susceptibility of IgG from different donors to oxidation, all of which also have a great influence on inverting the system to its basic state (different polyclonal IgGs).

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CONCLUSIONS

Redox-reactive antibodies are an extremely labile fraction of natural antibodies and all conditions influencing their activity are not yet determined. Oxidation, as a possible chemical transformation, triggered at low pH, could be just one of the possibilities of how to reveal the special subset of antibodies with increased specificity to several autoantigens.

We conclude that lower pH during isolation gives a higher yield, but alters the antibody molecule so much that they bind to some autoantigens.

Results of different immunological tests (aβ_2-GPI, aCL and aCCP ELISA) confirmed that, a) acetic acid with pH lower than 3.0 enables effective elution conditions, but it alters antibody binding properties; b) alterations in an antibody molecule are dependent not only on elution pH, but also on elution buffer composition (acetic acid of different pH, glycine-HCl buffer); c) the use of elution solutions with high buffer capacity is preferred when samples are expected to be further tested in experiments for susceptibility to redox reactions. We assume that the isolation of class G antibodies from sera following alternative protocol (the use of acetic acid, pH 2.4, alone) is also effective, but it is completely unuseful for studies in which redox-reactive antibodies may be involved.

Therefore, certain precautions are needed from isolation to the storage procedure of IgG fractions to preserve their original immunoreactivities. The use of high capacity elution buffers is preferred to mono component buffers because it prevents the possible specificity alteration of the subset of natural (redox-reactive) antibodies during isolation.

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