



Heterogeneous antigen recognition behavior of induced polyspecific antibodies

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ABSTRACT

Polyspecific antibodies represent a significant fraction of the antibody repertoires in healthy animals and humans. Interestingly, certain antibodies only acquire a polyspecific antigen-binding behavior after exposure to protein-modifying conditions, such as those found at inflammation sites, or used in small- and large-scale immunoglobulin purification. This phenomenon is referred to as “cryptic polyspecificity”. In the present study, we compare the potential of different chemical agents to induce IgG polyspecificity. Depending on the treatment used, quantitative and qualitative differences in the recognition of individual antigens from a standard panel were observed. Antibodies with cryptic polyspecificity utilized common mechanisms for the recognition of structurally unrelated antigens when exposed to a particular inductor of polyspecificity. Our study contributes to the understanding of the mechanisms underlying the cryptic polyspecificity.

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1. Introduction

A large proportion of circulating antibodies (Abs) in healthy individuals as well as in animals are polyspecific, i.e. single Ab molecules are able to recognize specifically various structurally unrelated antigens [1,2]. Such promiscuous antigen-binding was suggested to represent a mechanism of diversification of Ab repertoires, additional to that achieved by genetic processes of V-gene rearrangement and somatic mutation [3]. The pool of circulating Abs also includes Abs that are not polyspecific in their native form but acquire a promiscuous antigen-binding potential only after exposure to factors that affect protein stability [4]. Thus, a transient exposure of certain immunoglobulins (Igs) to low pH (pH ≤ 4), chaotropic agents or high salt concentration buffers has been shown to induce Ab polyspecificity [4–8]. Interestingly, the exposure of Igs to the above-mentioned protein-destabilizing conditions is part of the Ig purification processes, both in routine research laboratory practice and during the industrial production of several licensed therapeutic immunoglobulin preparations for intravenous administration (IVIg) [9]. This may explain, at least

in part, the observation that the antigen-binding reactivity of the native IgG antibodies in serum is considerably lower than that of the purified IgG fraction, isolated from the same serum [10–12]. The contact of the sensitive fraction of Igs with aggressive agents that are released *in vivo* in sites of inflammation or of tissue damage has also been shown to induce polyspecificity. Thus, exposure of certain Abs to reactive oxygen species, to iron ions or to heme as well as other oxidants results in the appearance of novel antigen-binding specificities [13–17].

Studies on the mechanisms of induced Ab polyspecificity have indicated that diversification of Ab specificity is accompanied by changes in the structural flexibility of the antigen-binding sites [7,15,18]. Little is known, however, on the effects of protein-destabilizing and redox agents at the level of antigen recognition. It thus remains unexplored whether the induction of polyspecificity by chemically different agents (redox, acidic pH, chaotropic) results in similar changes in the antigen-binding or whether treatment by each individual agent induces an unique set of new antigen-binding specificities. Moreover, it is not known whether the molecular mechanisms of recognition of structurally unrelated antigens by induced polyspecific Abs is unique or whether it depends on the antigen recognized.

In the present study, we compared the effects of different conditions known to induce *in vitro* Ab polyspecificity. It has been found that depending on the chemical nature of the treatment of the pooled IgG preparations, the recognized array of proteins by

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polyspecific Abs differs. Thermodynamic analyses have demonstrated that the recognition of structurally unrelated proteins by polyspecific ferrous ions-exposed IgG is governed by similar molecular mechanisms. Deciphering the mechanisms of acquired polyspecific antigen recognition by Abs should provide important insights towards the understanding of the biological relevance of this phenomenon in physiology as well as in pathological conditions.

2. Materials and methods

2.1. Ig preparations

Human therapeutic intravenous Ig preparations (IVIg) Intraglobin F and Intratect (Biotest AG, Dreieich, Germany) were used throughout the study. Intraglobin F and Intratect are produced without the use of an acidic pH blood-protein fractionation step. IVIg preparations were dialyzed against phosphate buffer saline pH 7.4 before use.

2.2. Treatment of Ig preparations

2.2.1. Exposure to ferrous ions

Iron (II) sulfate (Sigma–Aldrich) was dissolved in deionized water to 10 mmol/L. IVIg (50 μ mol/L) was exposed to 500 μ mol/L final concentration of Fe^{2+} at 4 °C for 30 min.

2.2.2. Exposure to heme

The oxidized form of heme (Hemin, Fluka), was dissolved to 1 mmol/L in dimethyl sulfoxide. IVIg (10 μ mol/L in PBS) was exposed to 20 μ mol/L final concentration of hemin at 4 °C for 30 min.

2.2.3. Exposure to hypochlorite

Sodium hypochlorite solution (Sigma–Aldrich) was diluted in deionized water to 16.7 mmol/L. IVIg (10 μ mol/L in PBS) was exposed to 167 μ mol/L of hypochlorite at 4 °C for 30 min before being used in the analyses.

2.2.4. Exposure to pH 2.8

IVIg was diluted to 50 μ mol/L in 0.2 mol/L glycine, pH 2.8. The preparation was incubated for 5 min at room temperature before adjusting the pH to 7.2 by adding aliquots of 3 mol/L solution of Tris pH 9. The acid buffer-exposed Igs were then dialyzed against PBS.

2.2.5. Exposure to urea

IVIg (50 μ mol/L in PBS) was dialyzed against excess of urea (6 mol/L in PBS) for 3 h at 4 °C. IVIg was then exhaustively dialyzed against PBS.

2.3. Enzyme-linked immunosorbent assays

Ninety-six well polystyrene plates (Nunc, Roskilde, Denmark) were coated for 2 h at 25 °C with 20 μ g/mL of human factor IX (FIX, Wyeth, La Défense, France), human factor VIII (FVIII, Helixate®, BayerPharma, Lille, France), human von Willebrand factor (VWF, LFB, Les Ulis, France), human C-reactive protein (CRP), human C3 (both from Calbiochem), human factor H (FH), human factor B (FB, both from Complement Technology Inc, TX, USA), porcine thyroglobuline (Tg), rabbit tubulin, bovine myelin basic protein (MBP) and human hemoglobin (Hb, all from Sigma–Aldrich) in PBS. Plates were blocked for 1 h with 0.25 % v/v tween 20 in PBS at 25 °C. The native and the treated IVIg preparations were diluted to 1 μ mol/L (150 μ g/mL) and incubated for 2 h at 25 °C. After washing with PBS 0.05% tween 20, mouse anti-human IgG antibody (heavy-chain specific) coupled to horseradish peroxidase

(Southern-Biotech, Birmingham, AL) was added and incubated for 1 h at 25 °C. Immunoreactivities were determined by using the *o*-phenylenediamine substrate (Sigma–Aldrich).

2.4. Kinetic and thermodynamic analyses

The kinetic parameters characterizing the binding of ferrous ions-exposed IVIg to VWF, tubulin and myelin basic protein (MBP) were determined by surface plasmon resonance (BIAcore 2000, GE Biacore, Sweden). Proteins were immobilized on CM5 chips by amino-coupling (Biacore). In brief, proteins were diluted in 5 mmol/L maleic acid, pH 5, to a final concentrations of 100, 40 and 5 μ g/mL, respectively, and injected on pre-activated sensor surfaces. The experiments were performed by using HBS-P (0.01 mol/L Hepes, pH 7.2, 0.15 mol/L NaCl, and 0.005% v/v tween 20). Ferrous ion-exposed IVIg were injected over the sensor surface at 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 μ mol/L with a flow rate of 10 μ L/min for 4 min. The dissociation phase of the interaction was then monitored for 5 min. Regeneration was performed after each injection by exposing the sensor surface for 30 s to a solution containing 50 mmol/L of NaOH and 500 mmol/L of NaCl. The BIAevaluation software (version 4.1, Biacore) was used for the calculation of the kinetic rate constants. Evaluation of the thermodynamic parameters of the interactions between Fe(II) -exposed IVIg and proteins was performed as described before [7]. Briefly, all kinetic measurements were performed at 10, 15, 20, 25, 30 and 35 °C. Kinetic rate constants obtained at various temperatures were used to build Arrhenius plots. The values of slopes of the plots were calculated by linear regression analysis using GraphPad Prism v.4 software (GraphPad Prism Inc.) and subsequently substituted in the Arrhenius equation:

$$Ea = -\text{slope } R,$$

where the “slope” = $\partial \ln k_{\text{on/off}} / \partial (1/T)$, and Ea is the activation energy and R is the universal gas constant ($R = 8.3144 \text{ J mol}^{-1} \text{ K}^{-1}$). The enthalpy, entropy and Gibbs free energy changes characterizing the association or dissociation phases were calculated using the following equations:

$$\Delta H = Ea - RT$$

$$\ln(k_{\text{on/off}}/T) = -\Delta H/RT + \Delta S/R + \ln(k'/h)$$

$$\Delta G = \Delta H - T\Delta S,$$

where T is the temperature in the Kelvin scale, k' is the Boltzman constant and h is the Planck's constant.

3. Results and discussion

3.1. Preferential antigen recognition by IgG antibodies with cryptic polyspecificity

The exposure of certain Abs to protein-modifying agents of different chemical nature has been reported before to induce a diversification of their antigen-binding specificities. We compared the effects of exposure of human pooled IgG (IVIg) to various agents on its binding to a panel of 11 structurally unrelated protein antigens. The latter included proteins from the coagulation cascade (FVIII, FIX, VWF), from the complement system (FB, FH, C3), from the acute phase response (CRP) and cellular proteins (Tg, tubulin, MBP and Hb). IVIg was exposed to three classes of chemical agents – redox agents (heme, ferrous ions and hypochlorite), chaotropic molecules (urea) as well as to pH 2.8 buffer. Igs may be exposed to any of these chemical agents and conditions at sites of inflammation or as a part of their purification process.

Exposure of IVIg to heme resulted in a considerable increase of the binding to all studied proteins (Fig. 1A). In contrast, other bio-

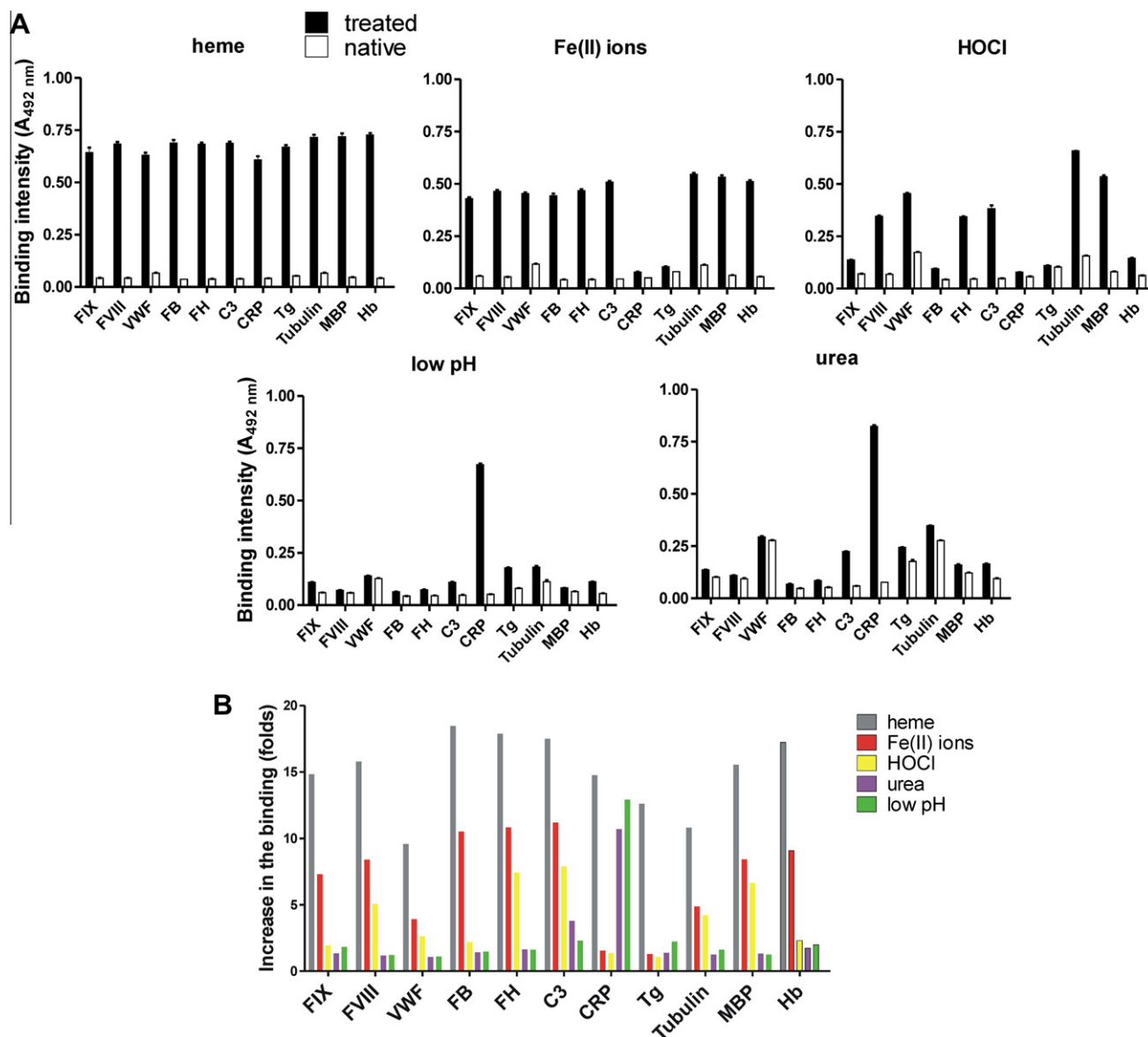


Fig. 1. (A) Induction of polyspecificity of IVIg towards a panel of self-proteins by different agents. Profiles of the reactivity of native IVIg (white bars) and of IVIg exposed to heme, ferrous ions, hypochlorite, low pH or urea (black bars). Each bar represents the mean optical density values $\pm$ SD of triplicate wells in one of two ELISA experiments. (B) Score of the increase in the reactivity of IVIg after exposure to protein-modifying agents. The bars represent the ratio obtained after division of the binding intensity of pooled IgG after exposure to chemical agent by the reactivity of the native preparation. For the calculations, raw data depicted in Fig. 1A were used.

logically-relevant oxidants such as free Fe(II) ions and hypochlorite (HOCl) induced a more selective increase in the reactivity towards the same proteins (Fig. 1A). Thus, the exposure to ferrous ions markedly increased binding of sensitive Abs to all proteins except to CRP and Tg, while, in the case of HOCl, the increased Ab binding was restricted to FH, C3, tubulin, MBP and FVIII. Yet, another pattern of reactivity was obtained after the exposure of the IgG preparation to low pH or to urea; both treatments resulted in a considerable increase in the binding to CRP. The reactivity towards other proteins was affected only marginally (Fig. 1A).

Further, we compared the potential of the individual protein-destabilizing agents to modify the binding to each of the antigens tested. To this end, the increase in the antigen binding was expressed as the ratio of binding scored for the treated IVIg versus that scored for the native IVIg (Fig. 1B). The data indicate that heme is the most potent agent able to induce the appearance of novel specificities of IgG. The reactivity of Abs exposed to heme increased by more than ten folds. The exposure to ferrous ions resulted in a 5- to 10-fold increase in IgG reactivity, while the exposure to HOCl

induced a 2- to 8-fold increase. In contrast, urea and acid pH exhibited a much lower potential (in most of the cases less than 2-fold) to enhance binding of IgG, except in the case of CRP where the binding increased more than ten fold.

The qualitative and quantitative differences in recognition of antigens by Abs after the induction of polyspecificity by the different agents suggest the existence of several molecular mechanisms underlying the appearance of the novel antigen-binding specificities. This may reflect the different chemical nature of the agents used in the study. Importantly, the powerful effect of heme was achieved by use of only a 2-fold molar excess over IgG (one heme molecule per Fab fragment). In contrast, other oxidizing agents – ferrous ions and HOCl, although used at 10 and 16 molar excesses over IgG, failed to achieve the effect of heme. Heme is a macrocyclic compound containing a protoporphyrin ring and one coordinated iron ion. Thus, when treated with heme Igs are exposed to two-molar excess of iron atoms. At such molar excess, free ferrous ions influence only weakly the antigen-binding properties of Abs [15]. Interestingly, exposure of Igs to the protoporphyrin ring of

heme devoid of iron (hematoporphyrin IX) does not reveal novel antigen specificities [19]. These observations imply that heme utilizes a different mechanism from that used by Fe(II) ions to induce Ab polyspecificity. Indeed, mechanistic studies performed by using a model monoclonal Ab had suggested that different redox-active agents may induce polyspecificity of Abs by different mechanisms. Thus, the exposure to ferrous ions was accompanied by an increase in the flexibility of Ab paratope [15], whereas the exposure to heme was followed by a decreased conformational freedom of the paratope [18]. Heme a strongly polyreactive molecule, has been shown to bind to antibodies and to serve as a cofactor in modulating the antigen-recognition activity of the latter [18,20].

Hypochlorite is an oxidizing agent produced *in vivo* by activated neutrophils. Its mechanism of action includes chlorination of some of the amino acid residues in proteins, that may cause structural alternations [21]. The specific oxidizing activity of HOCl could explain the observed unique pattern of recognized antigens by HOCl-treated Abs.

The fact that exposure of IVIg to urea or to low pH resulted in similar effects may be explained by the similar way in which these factors influence the protein structure. Indeed, urea and buffers with extreme pH values are both known to possess the ability to unfold polypeptide chain [22,23].

A common characteristic of all the studied agents able to reveal novel antigen-binding specificities of Abs, is that they are all able to induced alterations in the conformation of proteins. Some of the Igs retain their native conformation after transient exposure to protein-destabilizing agents as for example elution of protein G columns with acidic pH (pH 2.8). However, transient destabilization of the protein structure of other Igs may allow a localized molecular reorganization of the variable regions resulting in conformational variants with altered (in our study enhanced) antigen-binding capacity. It remains unclear, however, why only a fraction of all circulating Abs is able to reorganize its variable regions after transient exposure to protein-destabilizing agents [4]. This difference might be due to the fact that mutated antibodies, that bind with high affinity to their cognate antigens, have, as a rule, rigid paratopes [24].

3.2. Physicochemical mechanism of antigen recognition by polyspecific IgG

Next, we studied whether the recognition of structurally unrelated antigens by induced polyspecific Abs occurs by a common mechanism. By using a biosensor technique (BIAcore), we investi-

gated the binding of ferrous ion-exposed IVIg to tubulin, MBP and VWF. The interaction of the native Abs with these proteins was undetectable under the conditions of the biosensor experiment (data not shown). In contrast, ferrous ion-exposed IVIg recognized all three proteins (Fig. 2 and Supplement Figure). In accordance with the ELISA data, the intensity of binding of IgG to MBP was higher than that to tubulin or VWF. As depicted on the latter figures, the binding to the proteins increased with an increase of the temperature. The complexes were very stable as made evident by the absence of dissociation over the time interval studied.

We calculated the kinetic parameters characterizing the binding of the polyspecific Igs to tubulin, MBP and VWF. The fact that a highly heterogeneous anylite was used (IVIg) does not allow the estimation of the absolute, but only of the apparent values of the kinetic parameters. It may also introduce some artifacts in the kinetic experiment. However, a study of the behavior of a system that closely represents the complete human IgG repertoire has the advantage of being more relevant to the *in vivo* events – e.g. upon exposure of circulating Igs to redox-active agents in sites of inflammation. The use of a polyclonal Ig preparation also allows to better understanding how the complex system of Abs organizes itself to bind to a single target. The use of pooled IgG was thus aimed at obtaining comparative information for the overall mechanism involved. The apparent association rate constants (k_a) for the interaction of IVIg with MBP, tubulin and VWF at 35 °C were $725.5 \text{ M}^{-1} \text{ s}^{-1}$, $169.5 \text{ M}^{-1} \text{ s}^{-1}$ and, $222.5 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The high stability of the complexes of ferrous ion-exposed Igs with the studied proteins precluded the estimation of the apparent dissociation rate constants. However, the value of the equilibrium dissociation constant is at least in the nanomolar range if a dissociation rate of $1 \times 10^{-4} \text{ s}^{-1}$ is considered.

Further, we studied the temperature sensitivity of the apparent k_a constants for the binding of IVIg to MBP, tubulin and VWF. The Arrhenius plots depicted in Fig. 3A indicate that the increase in temperature resulted in an increase in the k_a in all cases. The same tendency was observed in the case of another structurally unrelated protein – FB (data not shown). Interestingly, despite of the differences in the values of k_a , the temperature sensitivity of the interaction was identical for the three structurally unrelated proteins as can be deduced by the nearly identical slopes of the linear regression fits (Fig. 3A). The Arrhenius plots allowed the calculation of the changes in the thermodynamic parameters that characterize the interaction of ferrous ion-exposed IVIg to the studied proteins (Fig. 3B). These analyses indicate that interaction of Abs with induced polyspecificity with the three antigens under study

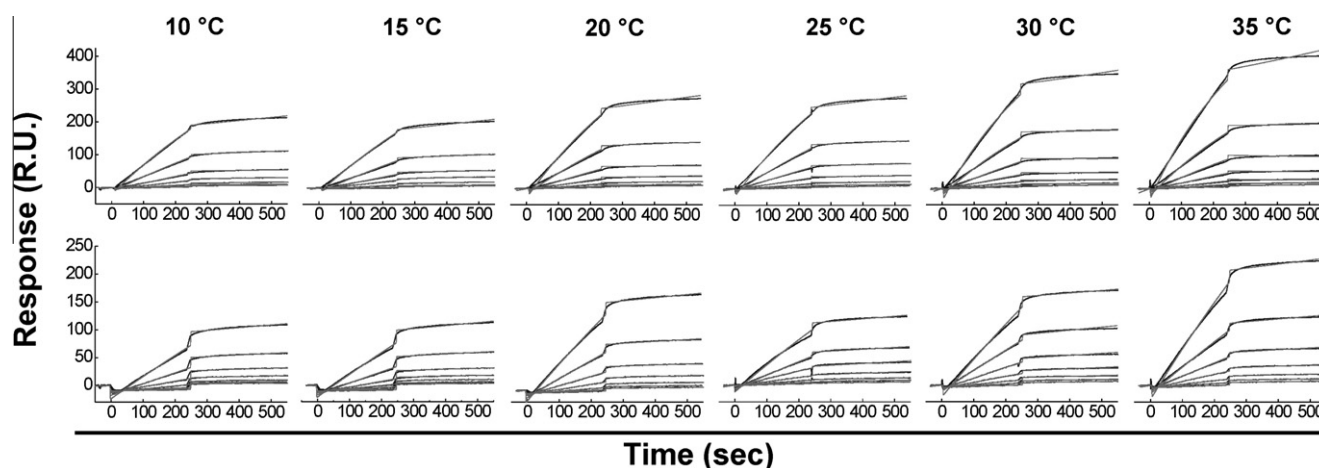


Fig. 2. Interaction profiles of ferrous ions-exposed IVIg with myelin basic protein and tubulin. The real-time profiles of the interaction of various concentrations of IVIg (5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 $\mu\text{mol/L}$) with immobilized myelin basic protein (upper row) and tubulin (lower row) were measured by SPR technology at the indicated temperatures.

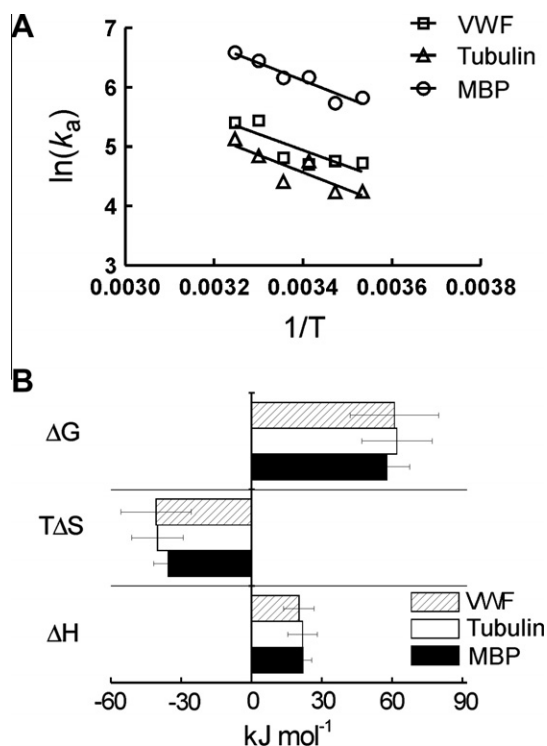


Fig. 3. Kinetics and thermodynamics of the interaction of IVIg with structurally unrelated proteins. (A) Arrhenius plots of the association rate constant for the interaction of ferrous ions-exposed IVIg with von Willebrand factor (open squares), tubulin (open triangles) and myelin basic protein (open circles). The reported values of the association rate constant were obtained by global analyses of the sensorgrams presented in Fig. 2 and in the Supplement Figure. The slopes of the Arrhenius plots were determined by linear regression analysis. (B) Thermodynamic analyses of the association of ferrous ions-exposed IVIg to structurally unrelated proteins. The bar graph depicts the changes in free energy, entropy and enthalpy during the association of ferrous ions-exposed IVIg to von Willebrand factor (dashed bars), tubulin (white bars) and myelin basic protein (black bars). The thermodynamic parameters were evaluated for a reference temperature of 298 K.

is accompanied by nearly identical changes in enthalpy, entropy and Gibbs free energy, irrespective of the target antigen (Fig. 3B).

The kinetics and the thermodynamic changes describe intermolecular interactions with respect to structural changes and the formation of non-covalent bonds. The very slow values of the apparent k_a observed in this study imply that the association with proteins is hampered by a high energetic barrier. This is confirmed by the thermodynamic analyses, which reveals very unfavorable changes in the free energy of association (apparent values of $57.7 \pm 9.8 \text{ kJ mol}^{-1}$, $62 \pm 15 \text{ kJ mol}^{-1}$ and $61 \pm 19 \text{ kJ mol}^{-1}$ for MBP, tubulin and VWF, respectively) resulting in unfavorable changes in entropy and enthalpy that characterize the studied interaction. The apparent values of enthalpy changes were nearly identical for the three studied antigens ($22 \pm 3.7 \text{ kJ mol}^{-1}$, $21.8 \pm 6.32 \text{ kJ mol}^{-1}$ and $20.2 \pm 6.5 \text{ kJ mol}^{-1}$ for MBP, tubulin and VWF, respectively). The entropy changes were also similar for the three different proteins ($-35.7 \pm 6.1 \text{ kJ mol}^{-1}$, $-40.2 \pm 11 \text{ kJ mol}^{-1}$ and $-40.8 \pm 15 \text{ kJ mol}^{-1}$ for MBP, tubulin and VWF respectively). High energetic barriers of protein–protein association (highly positive values of ΔG) result from a lack of structural complementarity of the interacting partners and characterize the presence of structural changes during the interaction process. The use of a heterogeneous system (i.e. polyclonal IgG) makes it practically impossible to predict the type of structural changes that occur in the interacting proteins. However, our data suggest that the recognition of structurally unrelated proteins by a pool of Abs exposed to a polyspecificity-inducing agent, is governed by the same biophysical mechanism. The use of a common molecular mechanism for the

recognition of different proteins, despite the enormous heterogeneity within the Ab pool, may be explained by the tendency of complex systems to organize to a simple statistically averaged behavior. Of note, the apparent changes in the thermodynamic parameters observed with IVIg are typical for Abs that interact with an induced-fit mechanism [25]. The increased molecular flexibility of pools of Abs may allow them to selectively accommodate various structural patterns and may thus explain the appearance of novel antigenic specificities and therapeutic benefit in various disease conditions [26].

4. Concluding remarks

A fraction of immunoglobulins in all healthy individuals diversifies its antigen-recognition repertoire upon the *in vitro* contact with aggressive redox agents released at sites of inflammation. It is tempting to speculate that the post-translational acquisition of novel antigen specificities represents an innate-like defense system that is at play before unfolding of the adaptive immune response.

In the presented study, we compared the ability of various agents to diversify the antigen-recognition repertoire of pooled IgG. The obtained results demonstrate that, depending on the chemical agent used, different intensities and different patterns of increased antigen-binding behavior by the human antibodies are observed. The molecular mechanisms responsible for the enhanced recognition of structurally unrelated antigens, elicited upon exposure to an individual studied protein-modifying agents are, however, identical.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.073.

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