Induced antigen-binding polyspecificity in human serum IgA

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Abstract: Recent studies have shown that polyspecific antibodies play an important role in the frontline defense against the dissemination of pathogens in the pre-immune host. Interestingly, antigen-binding polyspecificity can not only be inherent, but also acquired post-translationally. The ability of individual monoclonal IgE and IgG antibodies to acquire polyspecificity following contact with protein-modifying agents has been studied in detail. However, to the best of our knowledge this property of human IgA has previously been described only cursorily. In the present study pooled human serum IgA and two monoclonal IgA antibodies were exposed to buffers with acidic pH, to free heme or to pro-oxidative ferrous ions, and the antigen-binding behavior of the native and modified IgA to viral and bacterial antigens were compared using Western blotting and ELISA. We observed a dose-dependent increase in reactivity to several bacterial extracts and to pure viral antigens. This newly described property

of IgA may have therapeutic potential as has already been shown for pooled IgG with induced polyspecificity.

Keywords: Human serum IgA, monoclonal IgA, antibody specificity, induced polyspecificity

Declarations of interest: none

Introduction

The enormous diversity of antibody specificities is the result of complex genetic recombination and mutational processes. Epigenetic mechanisms can increase this diversity still further [1]. Naturally polyspecific antibodies, referred to also as polyreactive, multispecific, promiscuous or degenerate, are present in the blood of all healthy individuals [2]. While most IgM antibodies are known to be polyspecific, only a portion of IgE and IgG immunoglobulins possess this property. Scientific proof for the existence of polyspecific antibodies first came when it was demonstrated that some monoclonal antibodies could bind more than one structurally different antigen. The molecular mechanisms of polyspecific antigen binding are discussed in detail elsewhere [3].

Natural polyspecific antibodies play an important role as a frontline defense against invading pathogens, in maintenance of immune homeostasis, in the clearance of senescent and damaged cells, and etc. [4]. Twenty years ago it was shown that in addition to innate (natural) polyspecificity, some immunoglobulins can acquire this property after exposure to chaotropic agents, buffers with low pH, high salt solutions, or pro-oxidative molecules [5]. We hypothesized that this induced specificity may be observed not only *in vitro*, but also *in vivo* – at sites of inflammation where various aggressive protein-modifying molecules are released. Indeed, it was later demonstrated that induction of polyspecificity through exposure of IgG molecules to protein-modifying agents is not a laboratory artifact, but can also occur *in vivo* [6]. Free heme and ferrous ions are known to be released *in vivo* at sites of inflammation, trauma, as result of intravascular hemolysis, and etc. The ability of heme to induce IgG antigen-

binding polyspecificity has been reported by J. A. McIntyre *et al*. [7]. Moreover, parasite invasion may induce IgG and IgM polyspecificity towards mammalian cells [8], and exposure of immunoglobulins to histones can also trigger their polyspecificity [9].

Our previous studies have shown that the exposure of human IgG to heme, to ferrous ions or to buffers with acidic pH results in the induction of different degrees of polyspecificity [1,10–12]. Pooled human IgG for intravenous administration (intravenous immunoglobulin, IVIg) with an enhanced ability to bind additional antigens has therapeutic potential. While the infusion of native (untreated) pooled human IVIg has no beneficial effect in mice with experimental sepsis, the same dose of ferrous ions-treated IVIg increases the animals' survival significantly [12]. Recently we have also shown that a monoclonal IgE antibody exposed to heme can acquire additional antigen-binding polyspecificity [13].

More serum and secretory IgA are produced per day in the human body than all antibodies of the other immunoglobulin classes taken together. While the role of secretory IgA in health and disease is quite well understood, that of the serum IgA is not so well elucidated. Interestingly, serum IgA has a complex role as it is responsible not only for anti-inflammatory, but also for pro-inflammatory responses *in vivo* (for review see [14]). The aim of our study was to formally prove the ability of heme, low pH buffers and Fe(II) ions to induce polyspecificity in human serum IgA.

2. Materials and Methods

2.1. Serum IgA preparations

Polyclonal human serum immunoglobulin A (IgA) was kindly provided by Dr. Srini Kaveri (INSERM U1138 Paris, France). Serum IgA, exposed to low pH during its fractionation was obtained from the biotechnology company Imtek (Moscow, Russia). Its exposure to heme was performed as described previously [15]. The exposure to pro-oxidative ferrous ions was carried out as follows: serum IgA (5 mg/mL) was incubated (at 4°C for 30 min) in freshly prepared 1 mM solution of $FeSO_4.7H_2O$ in PBST buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, and 0,1% Tween 20 (all from Sigma-Aldrich, USA). Next, the

IgA solution was dialyzed for 120 min. at 4°C against 1x phosphate–buffered saline (PBS) containing 5 μM ethylenediaminetetraacetic acid (EDTA) and finally against 1x PBS without EDTA (4°C, 24 h). The following buffers were used to evaluate the ability of low pH conditions to induce IgA polyspecificity: TRIS buffer (pH 7.0) and two glycine buffers (with pH 2.6 and with pH 4.0).

2.2. Cloning of monoclonal antibodies

Variable regions of the heavy and light chains of mAb21 and mAb72 originated from a previously described repertoire of human antibodies obtained by single-cell PCR from synovial tissue of rheumatoid arthritis patients [16]. Variable regions were cloned in IgA1 expression vectors by the In-Fusion technology (Takara) as per manufacturer's protocol. The expression vectors were kindly provided by Dr. Hugo Mouquet (Institute Pasteur, Paris France) [17].

2.3. Expression and purification of monoclonal antibodies

For each antibody, expression vectors for the heavy and light chain were co-transfected in the ExpiCHO cell line (Thermo Fisher Scientific, USA) using the manufacturer's protocol. In brief, 2.5 mL of a $6x10^6$ cells/mL suspension were co-transfected with a total of 2.5 µg plasmid DNA (1:1 molar ratio of heavy and light chain expression vectors). Transfected cells were incubated for 8 days at 37°C and 8% CO_2 with shaking (225 rpm). The supernatants were harvested by centrifugation at 3000 x g for 30 minutes at 4°C, filtered through a 0.22 µm syringe filter and diluted with 1 volume of PBS. For each antibody, 4 mL of 50% gel slurry of agarose-immobilized Jacalin (Thermo Fisher Scientific, USA) were loaded on a gravity column. After sedimentation the packed bed was first equilibrated with 10 mL of PBS. Diluted supernatants were loaded onto the columns and the unbound fraction was washed with 10 mL PBS. IgA antibodies were eluted with 10 mL elution buffer (0.1M alpha-D-galactose in PBS) in 2 mL fractions. IgA-containing fractions were pooled and dialyzed against PBS. Concentrations were estimated by spectrophotometry.

2.4. Preparation of antigen extracts for Western blotting

E. coli (BL21) and *S. aureus* (2879 M) were cultured on an orbital shaker in sterile LB liquid medium containing 10 g Tryptone, 10 g NaCl, 5 g yeast extract (all from Sigma-Aldrich, USA) prepared in distilled water up to 1 L, (pH was adjusted to 7.5) for 16–18 hours at 37°C, then centrifugated (at 14 000 rpm for 5 min.). After removal of the supernatant the pellet was resuspended in 5 mL of cell lysis buffer containing 30 mM NaCl, 50 mM, TRIS (pH 6.8), 5% glycerol, and 0.5% Triton X-100 (all from Sigma-Aldrich, USA), homogenized by sonication and centrifugated for 15 min at 15000 rpm. The lysate supernatants were used for Western blot analysis.

Cells from the human cancer cell line Colo205 were cultivated in complete RPMI-1640 medium (Thermo Fisher Scientific, USA) supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and 10% fetal bovine serum (Thermo Fisher Scientific, USA). The cells were detached from the culture flask using 0.25% (w/v) Trypsin/0.53 mM EDTA solution and resuspended (at $4x10^6$ cells/ml) in 0.5 mL of lysis buffer [50 mM TRIS base with pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, all from Sigma-Aldrich, USA] with constant agitation at 4°C for 30 min. The cells were then centrifugated (4°C, 20 min, 21 000 rpm) and the supernatant was aspirated, placed in a fresh tube, kept on ice and used for the Western blot analysis.

2.5. Western blotting

The proteins from the lysates were transferred to nitrocellulose membranes (Bio-Rad, USA) using standard Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) in TRIS/Glycine buffer [25 mM TRIS base (pH 7.8-8.5), 190 mM Glycine, 0.1% SDS, and 20% Ethanol, all from Sigma-Aldrich, USA] for 1.5 h at 400 mA. The nitrocellulose membrane (0.2 µm, Bio-Rad, USA) was blocked with 5% non-fat dried milk in TBST buffer [50 mM TRIS base (pH 7.5), 150 mM NaCl, 0.05% Tween-20, all from Sigma-Aldrich, USA] for 1 h at room temperature to prevent non-specific binding. The membrane was washed 5 times with TBST (for 5 min.), cut into stripes and incubated with the native or the modified (with heme, Fe(II) or acidic pH) antibodies for 2 h at room temperature and washed with TBST five times. Next,

the stripes were incubated with a Goat Anti-Human IgA alpha chain (HRP-conjugated) antibody (ab6858, Abcam, UK) (1:10000), washed with TBST 5 times and twice with TBS, and then incubated in 100 mM TRIS-solution (pH 8.0) containing 0,8 mM iodophenylboronic acid, 1.25 mM luminol and 30% hydrogen peroxide. The stripes were then placed in a digital scanner for chemiluminescent blots (LI-COR Biosciences, UK). The results were analyzed using the LI-COR Biosciences software Image Studio (LI-COR Biosciences, UK).

2.6. Enzyme-linked Immunosorbent Assay (ELISA)

To assess the binding activity of serum IgA to viral antigens 96-well polystyrene plates (Corning® 96-well Clear Polystyrene High Bind Stripwell™ Microplate, Corning, USA) were coated overnight at room temperature with the following antigens: Hepatitis C virus NS3 antigen (HCV), Hepatitis D virus antigen (HDV), and the p24 antigen of human immunodeficiency virus type 2 (HIV) (the antigens were kindly provided by RPC Diagnostic Systems, Russia). After blocking with PBS containing 5% BSA, the wells were washed 4 times with PBS containing 0.05% Tween 20 and incubated with increasing concentrations of native or modified IgA for 1 h at 37°C (in PBS containing 0.05% Tween 20 and 1% BSA). After washing, the plates were incubated with a Goat anti-human IgA alpha chain (HRP-conjugated) antibody (ab6858, Abcam, UK) for 1 h, followed by treatment with 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich, USA). Optical density values were read at 450/620 nm. The results were analyzed as the ratio between the optical density of native IgA and IgA exposed to protein-modifying molecules. A ratio equal or higher than 2 was considered as a significant increase in polyspecificity.

3. Results

3.1. Exposure of serum immunoglobulin A and monoclonal IgA to free heme increases binding to antigens

A variety of pathological processes cause degradation of hemoglobin and myoglobin, and release free heme into the circulation [18]. Several plasma proteins - hemopexin, albumin, haptoglobin and some lipoproteins are able to bind heme and eliminate it from the plasma [19].

However, when the quantity of released heme is above the binding capacities of its scavengers, the residual free heme can bind to immunoglobulin molecules and change the nature of the noncovalent forces responsible for their antigen binding [20]. We observed that the exposure of serum IgA to 0.6 µM and 1.2 µM heme slightly increases its binding to antigens from *E. coli* and Colo205 cells (Figure 1A). Similarly, following exposure to heme the monoclonal antibodies mAb21 and mAb72 recognize novel antigens from *E. coli*, *S. aureus* and Colo 205 cells. (Figure 1 C, D). Exposure to heme failed to change the ability of serum IgA to recognize and bind *S. aureus* antigens (Figure 1A) and did not affect its reactivity to the purified viral antigens tested (Figure 1B). In addition, we wanted to further investigate whether or not heme can induce binding of mAb21 and mAb72 to human self-antigenes using ELISA (Supplementary method). Indeed, we found the appearance of new reactivities toward various autoantigens (Supplementary Figure S1).

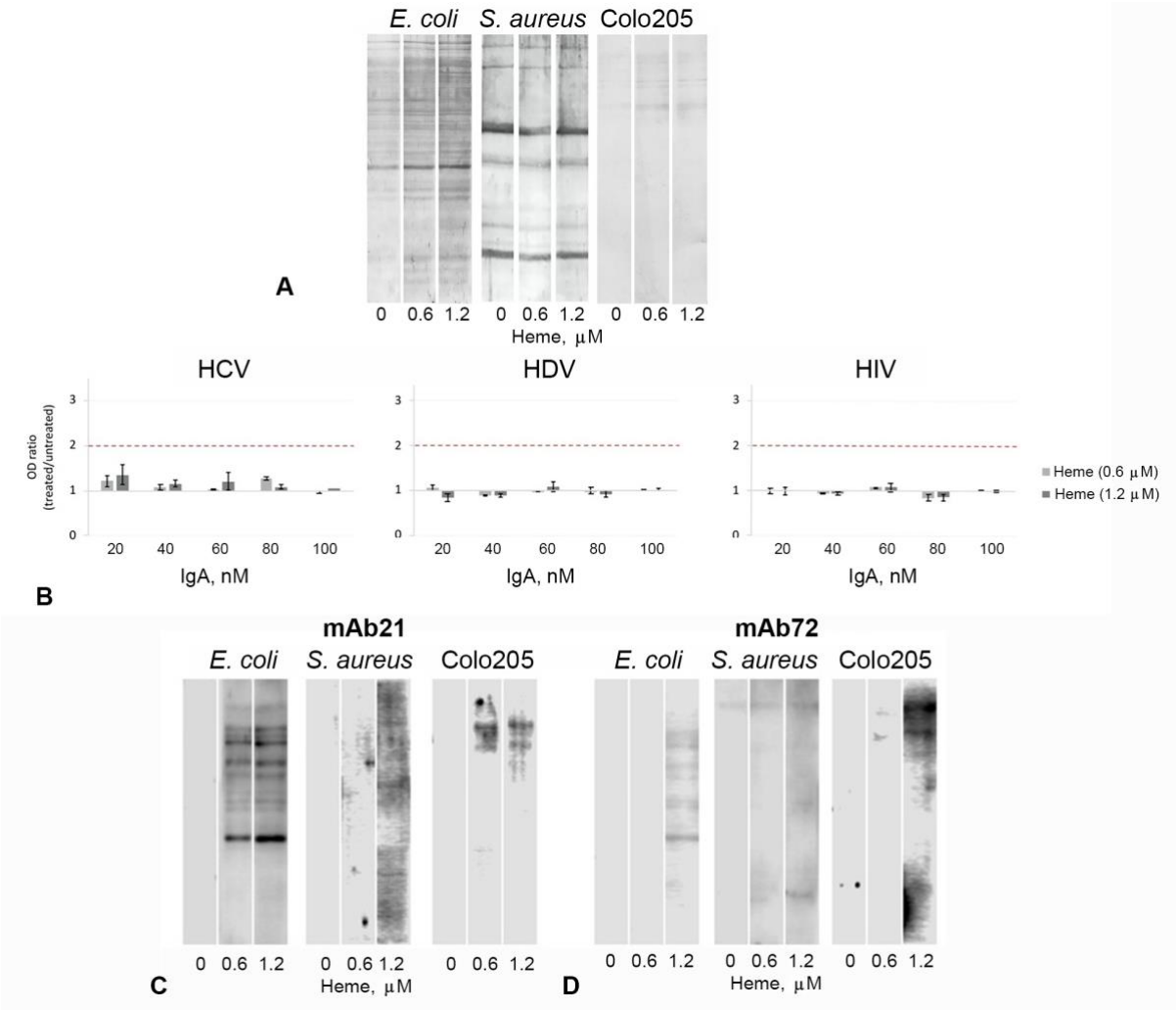

Figure 1. Increased immunoreactivity of human serum IgA and monoclonal antibodies after exposure to heme. (A) Exposure of human serum IgA to heme slightly increases its binding to antigens from Colo205 and *E. coli*, but not from *S. aureus* (Western blot analysis). (B) Heme-treated IgA does not alter the recognition and binding to selected purified viral antigens as shown by ELISA. The data represents mean ±SEM. Exposure of two monoclonal antibodies (C) mAb21 and (D) mAb72 to heme increases their binding to *E. coli*, *S. aureus* and Colo205 antigens.

3.2. Pooled human serum IgA become polyspecific after exposure to Fe(II) ions monoclonal IgA free heme

It is well known that transition metal ions possess a potent pro-oxidative potential and it has been shown earlier that the pro-oxidative potential of Fe(II) ions causes enhanced antigen-binding reactivity in ferrous ion-sensitive IgG [12]. It has been observed that the exposure of

IgG to Fe(II) ions results in a strong increase in their antigen-binding polyspecificity and these modified immunoglobulin preparations have anti-inflammatory therapeutic potential [12,21]. We were interested to explore whether or not Fe(II) ions have the same effect on serum IgA. Indeed, our results show that exposure to Fe(II) ions (concentrations ranging from 25 to 400 μM) increased the binding activity of IgA to *E. coli* and Colo205 antigens in a dose-dependent manner (Figure 2A). Similarly to the results obtained for the heme-treated group, we failed to detect a change in binding activity to *S. aureus* antigens (Figure 2A). Exposure to two different doses of Fe(II) ions (25 and 100 μM) induced binding to viral antigens and reached above the 2-fold threshold (see Materials and Methods) at the highest IgA concentration (100 nM; Figure 2B). However, results obtained for monoclonal antibodies shown that incubation with Fe(II) did not change their antigen-binding properties (Supplementary Figure S2).

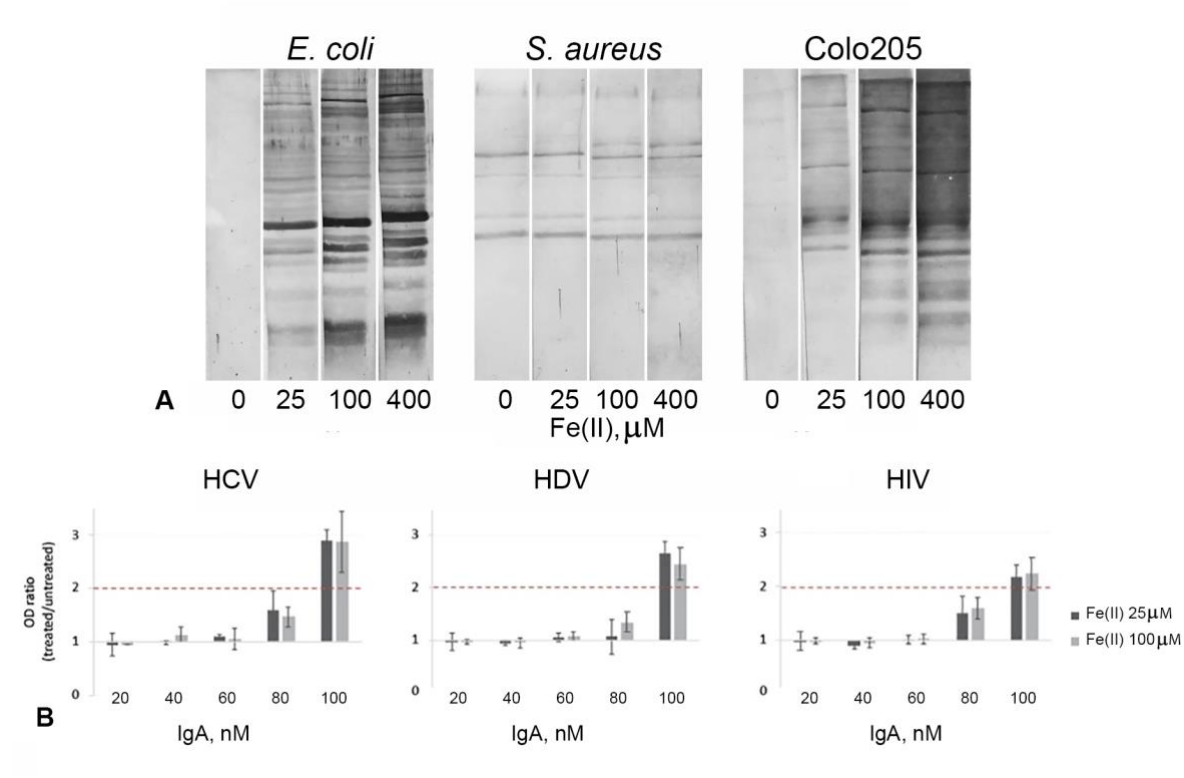

Figure 2. Increased immunoreactivity of human serum IgA after exposure to Fe(II) ions. (A) Western blot analysis shows that the polyspecificity of serum IgA exposed to increasing concentrations of Fe(II) ions induces the binding to *E. coli* and Colo 205 antigens, but not to *S. aureus* antigens. (B) Data from the ELISA analyses shows that after treatment with Fe(II) ions the same polyclonal IgA at higher concentrations (80 and 100 nM) acquires enhanced binding ability to single viral antigens. The data represents mean ±SEM.

3.3. Exposure to buffers with low pH induces IgA polyspecificity

Low pH treatment changes the structure of the IgG molecules and causes an enhancement of IgG antigen-binding polyspecificity [11]. In order to delineate whether or not acidic buffers have a similar effect on the antigen-binding behavior on serum IgA its solutions of different concentrations were exposed to buffers with different pH and then measured the binding activity to bacterial or viral antigens by Western blot or ELISA, respectively. We observed improved binding of IgA after treatment with acidic buffers only in the pH=4 group. Both the repertoire of detected antigens (shown as the number of bands) and the intensity of staining were increased after treatment of the IgA (Figure 3A). We also observed a modest increase in the IgA antigen-binding ability to individual viral antigens after exposure to an acidic buffer

with pH=4.0. Exposure to the buffer with an even lower pH (pH=2.6) reduced the antigen binding capacity (Figure 3A,B). And we did not observe any changes in antigen-binding properties of Fe(II)-treated monoclonal antibodies mAb21 and mAb72 (Supplementary Figure S3).

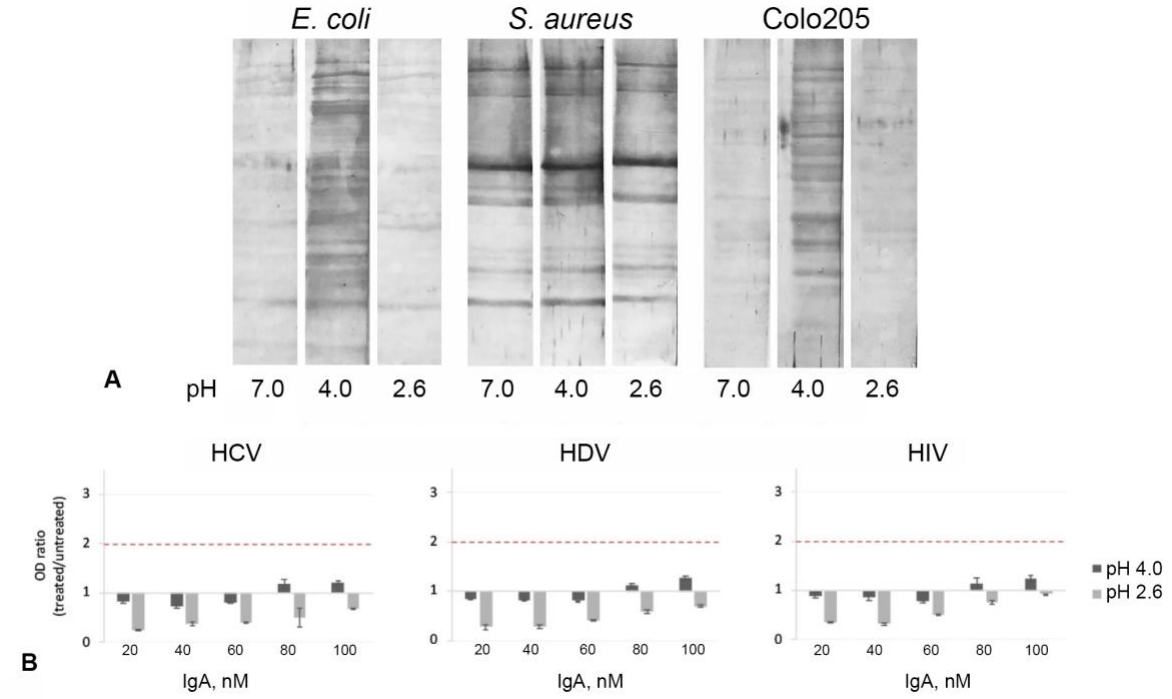

Figure 3. Increased immunoreactivity of human serum IgA after exposure to buffers with low pH. (A) Exposure of human serum IgA to buffers with pH 4.0 but not pH 2.6 results in an increase in the binding to bacterial- and auto-antigens; (B) ELISA results show that after treatment with a buffer with pH 4.0 polyclonal IgA at 12 mg/mL or higher concentration demonstrates insignificant increase in binding to individual viral antigens. Exposure to the buffer with pH 2.6 reduced its antigen-binding ability. The data represents mean ±SEM.

4. Discussion

We have previously described in detail the mechanisms and biological consequences of inducing additional polyspecificity in a human monoclonal IgE antibody (by exposure to heme only [13]), as well as in polyclonal human IgG preparations including one used in the clinic for the treatment of patients (IVIg). The latter has been exposed to free heme [1,20,22], to low

pH buffers [5,11] as well as to ferrous ions [21]. The phenomenon of induced polyspecificity has been studied mostly *in vitro*. Furthermore, local concentrations of free heme, pro-oxidative ferrous ions and reactive oxygen species at sites of inflammation and severe trauma could reach levels that are sufficient to modify the antigen-binding behavior of circulating immunoglobulins [6]. IVIg preparations were assayed for the treatment of sepsis and related systemic inflammatory response syndromes [23,24]. However, the higher-quality randomized clinical trials failed to prove any beneficial effect [25]. We have also demonstrated that while the administration of unmodified IVIg had no effect on survival of mice with sepsis and two types of severe aseptic response syndromes, the survival of animals treated by IVIg with additional induced polyspecificity was significantly improved [21].

The present study supports our hypothesis that the exposure of human serum IgA to heme, ferrous ions or to low pH may modulate its antigen-binding specificity in the same manner as reported earlier for IgG. It also suggests that serum IgA with induced polyspecificity may have a therapeutic potential as has been previously reported for IgG [22]. The most pronounced effect was observed for pooled human serum IgA treated with ferrous ions. Heme treatment also caused changes in the binding of the pooled serum immunoglobulin A, but the effect was not as pronounced as for ferrous ions. However, the consequences of monoclonal antibodies exposure to heme were so significant that they began to bind even individual autoantigens. At the same time, we did not observe any effect for monoclonal antibodies after treatment with acidic pH and Fe(II). This can probably be explained by the peculiarities of individual monoclonal antibodies. It's possible that different monoclonal antibodies may be sensitive to different types of treatments (Fe(II), low pH or heme), but this hypothesis remains to be tested.

The method of choice for the rapid purification of immunoglobulins of human and animal origin is based on the specific binding of its molecules to immobilized Protein A and Protein G. The elution step is performed by washing of the respective immunoaffinity columns with low pH buffer. Most of the commercially available purified human serum IgA and secretory IgA are produced this way. Our studies have shown that this treatment causes enhancement of

IgA antigen-binding polyspecificity. The subsequent use of these modified immunoglobulin molecules in different experiments will inevitably result in biased results (Supplementary Figure S4). This suggests that this approach should not be used and should perhaps be replaced by milder techniques (e.g. using the gentle Ag/Ab elution buffers).

It is known that monomeric IgA plays a significant role in maintaining homeostasis through the FcαRI receptors, inducing immune cells and pro-inflammatory responses resulting in elimination of pathogens and cancer cells [26,27]. At the same time excessive formation of IgA immune complexes can result in heightened neutrophil activation and autoimmune damage [26,28]. Nevertheless, it is important to keep in mind that while expansion of the repertoire of heme-treated antibodies may provide additional protection against pathogens, it may also contribute to the pathogenesis of inflammation and autoimmunity [20]. It is important to understand that the role of serum IgA in the immune response has not been fully appreciated and the results obtained indicate that the ability of serum IgA to change antigen-binding behavior must also be considered.

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