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Influence of isotypes of disease-associated autoantibodies on the expression of natural autoantibody repertoires in humans

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Abstract

Current understanding of immune network interactions mediated by immunoglobulins focuses on the role of idiotypes expressed on antibody variable regions. Idiotype interactions account significantly for the functional integrity of natural self-reactive antibody repertoires, whereas immunoglobulin isotypes are not considered to direct natural autoimmunity. Autoimmune thrombocytopenic purpura (AITP), a bleeding disorder caused by clonally restricted platelet-specific autoantibodies of the IgM or IgG isotype, is an excellent model to investigate the impact of isotype differences of immunoglobulins on the selection of natural self-reactive antibody repertoires in humans. Using specific analytical techniques to characterize the natural self-reactive antibody repertoire (i.e. quantitative immunoblotting, affinity biosensor technology), we here demonstrate that isotype differences of disease-associated autoantibodies are associated with altered natural self-reactive antibody repertoires in humans. Our data suggest that regulation of natural autoreactivity by antibody isotype might occur under certain conditions. The control of natural self-reactive antibody repertoires by immunoglobulin isotypes at a supraclonal level may provide a structural basis for non-organ-specific broad alterations of natural self-reactive antibody repertoires in organ-specific autoimmune diseases.

Keywords: Self-tolerance; Natural autoantibodies; B cells; Immunoglobulin isotypes; Immunoglobulin idiotypes

1. Introduction

Research of the past years provided increasing evidence that natural autoreactivity is essential for establishing and maintaining immunotolerance [1–3]. Natural autoreactivity is mediated by autoreactive T cells, B cells and immunoglobulins. Immunoglobulins express antigenic determinants on their variable regions, the idiotypes. Idiotype interactions account significantly for the functional integrity of natural self-reactive antibody repertoires, whereas immunoglobulin heavy-chain constant regions, the isotypes, are not considered to direct natural autoimmunity. The existence of a natural autoantibody toward a definitive self-antigen depends on positive selection of autoreactive B cells by their corresponding self-antigens [4–6]. In the case the self-antigen is an immunoglobulin molecule, the selection of the corresponding autoreactive B cell clone occurs by idiotype-anti-idiotype interactions of the immunoglobulin molecule and the corresponding B cell receptor (BCR) [7–9]. Immunoglobulin isotypes do not contribute to the selection of natural self-reactive B cells specifc for immunoglobulins in humans. However, the individual B cell and the individual immunoglobulin molecule are merely the single components of a complex system that might function in its complexity according to rules different from principles guiding the interactions of the single components. We were interested in the question whether immunoglobulin isotypes might contribute to the selection of natural self-reactive antibody *repertoires*, such that natural autoantibodies, selected at the level of idiotype-anti-idiotype interactions between an immunoglob-

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ulin molecule and its corresponding BCR, are bundled by isotype-dependent mechanisms to act as an entirety, i.e. the natural autoantibody *repertoire*. Dependence of natural self-reactive antibody repertoires on immunoglobulin isotypes might open a way to understand the control of natural autoimmunity at a supraclonal level, and provide new insights into functional mechanisms of immunoglobulin-dependent immunoregulation in humans.

Analysis of natural autoantibodies has so far been performed at the level of investigation of single components of the biological system, such as isolated autoantibodies and purified autoantigens or recombinant peptides. Quantitative immunoblotting followed by multiparametric statistical analysis of the data is a technique developed to investigate complex interactions of complex antibody mixtures such as human plasma toward complex antigen mixtures extracted from human cells and tissues [10,11]. The experimental design makes it possible to compare changes of complex interactions in dependence of variables such as time, and principally allows to depict functional dynamics of the investigated system. Taking autoimmune thrombocytopenic purpura (AITP) mediated either by autoantibodies of isotype IgM or of isotype IgG as a disease model, we have used this methodological approach to get access to the question whether isotypes of immunoglobulins contribute to the selection of natural antibody repertoires. AITP is a bleeding disorder caused by clonally restricted self-reactive antibodies with specificity for platelet glycoproteins [12,13]. Thus, the analysis of autoantibody repertoires directed toward platelets depicts shifts of disease-associated autoantibody repertoires, and the analysis of autoantibody repertoires directed toward antigens derived from organs such as kidney, liver or red blood cells depicts shifts of natural autoantibody repertoires.

2. Material and methods

2.1. Patients' characteristics

EDTA plasma samples were obtained from 21 adult patients with AITP (11 male patients, 10 female patients, mean age 54 years, range 23–86 years). Patients presented with thrombocytopenia. The diagnosis of AITP was made on the basis of verification of platelet reactive autoantibodies free in plasma and bound to the platelet surface. Autoantibodies eluted from the platelet surface were available in addition to antibodies derived from plasma in some of the cases. Autoantibody glycoprotein specificity and isotype was determined by the platelet immunofluorescence test [14]. They were confirmed by the MAIPA assay in the case of IgG antibodies [15], and by a commercially available ELISA (GTI, Brookfield, USA) in the case of IgM antibodies. Seven patients were shown to exhibit anti-platelet antibodies of isotype IgG only, and seven patients to exhibit anti-platelet antibodies of isotype IgM only. The concentration of overall IgM and IgG in patients' samples were determined by ELISA. Concentrations of IgM as well as concentrations of IgG between AITP subgroups did not differ significantly ($p > 0.01$, Mann–Whitney U test). None of the patients had received treatment prior to the collection of blood samples. Studies were approved by the ethics commission at the University of Muenster.

2.2. Investigation of antibody reactivities by quantitative immunoblotting

Investigation of antibody reactivities by quantitative immunoblotting was performed according to recently published protocols [11,16]. In brief, the reactivities of IgM in plasma (20 μg/ml), IgG in plasma (200 μg/ml), and IgG purified from plasma by affinity chromatography on protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden; 200 μg/ml as determined spectrophotometrically at 280 nm) were analysed by a western blot technique using extracts of normal human platelets, red blood cells, liver, and kidney as sources of self-antigen. Protein extracts were subjected to preparative SDS-PAGE in 10% polyacrylamide. The amount of solubilized proteins subjected to electrophoresis ranged between 200 and 600 μg/gel, depending on the tissue or cell extract, and was maintained constant for a given extract in all experiments. Following electrophoresis, the proteins were transferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) for 1 h at 0.8 mA/cm^2 using a Semi Dry Electroblotter model A (Ancos, Hϕjby, Denmark). Membranes were blocked for 1 h at room temperature with PBS containing 0.2% Tween 20. Patients' plasma was then incubated with the membranes following the addition of one sample per slot in a Cassette Miniblot system (Immunetics Inc., Cambridge, MA). The membranes were incubated for 4 h at room temperature, washed and revealed with μ-chain-specific secondary rabbit anti-human IgM antibody or with γ-chain-specific secondary rabbit anti-human IgG antibody coupled to alkaline phosphatase (Dako, Glostrup, Denmark) for 90 min at room temperature. Immunoreactivities were revealed using the NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) substrate (Sigma, St. Louis, MO). Quantitation of immunoreactivities was performed by scanning the membranes (SnapScan 600, Agfa Gevaert, NY). Blotted proteins were stained with colloidal gold (Protogold, BioCell, Cardiff, GB) and subjected to a second densitometric analysis to record the protein profile and to quantitate transferred proteins. Data were analyzed using a Power Mac G4 computer (Apple Computer Inc., Cupertino, CA) and the IGOR software (Wavemetrics, Lake Oswego, OR). Densitometric profiles of immunoreactivity were compared by referring to their corresponding protein profile following correction of the migration defects by superimposition of protein peaks. Normal human IgG for therapeutic intravenous use (IVIg, Venimmun®) was used as a standard for IgG. A standard for human IgM (IVIgM) was obtained by subjecting an IgM enriched therapeutic preparation of normal human immunoglobulin, Pentaglobin®, to size

exclusion chromatography on HiPrep Sephacryl S-200 (Pharmacia, Uppsala, Sweden). A sample of the reference IgM or IgG preparations was included in each blot, allowing to calibrate immunreactivities, to rescale different membranes transferred with a given protein extract, and to adjust for the intensity of staining of different membranes. Migration distance (*X*-axis) and optical density (*Y*-axis) were expressed as arbitrary units (AU). Migration distances of 200, 600 and 1000 AU corresponded to apparent molecular weight of 200, 65 and 20 kDa, respectively [17]. The assay was reproducible with a coefficient of variation of up to 10%. The 95% confidence interval of the mean area under the curve corresponding to each peak of immunoreactivity was 30% in the case of IgM [18] and 25% in the case of IgG [19], as calculated using Student's *t* test.

2.3. Multiparametric statistical analysis of the data

Multiparametric statistical analysis of the data generated by quantitative immunoblotting was performed using the IGOR software, including specially written software packages [20], and the StatView software. The details of analysis have been published elsewhere [10–21]. In brief, each densitometric profile of reactivity of antibodies of each individual was divided into sections corresponding to the individual peaks of immunoreactivity in each profile. The respective areas under the curve were calculated for each peak of reactivity for each individual and submitted to principal component analysis (PCA) [21]. PCA discriminates the repertoires of reactivities of antibodies from different individuals and summarizes, with minimum loss of information, the multidimensional information that is accounted for by the values of peak areas in all the sections, into a two-dimensional graph (factor 1 versus factor 2). In order to differentiate with high reliability between extracts which are target for the disease-associated autoantibodies (i.e. platelets) and those which are not (i.e. liver, kidney and erythrocytes), we performed the PCA on extracts of liver, kidney and erythrocytes as cumulative PCA, maximizing the number of dimensions contributing to the outcome of the result. In the present study, the amount of information taken into account by the PCA varies between 48 and 86%, depending on the tissue extracts. These percentages represent the eigen values that characterize each of the factors generated by the PCA. Each symbol in a PCA graph represents the repertoire of antibody reactivities of a single individual. The significance of the discrimination between antibody repertoires of groups of individuals was investigated by submitting the PCA data to linear discriminant analysis (LDA), and by subsequently comparing factors 1 of the LDA by means of a Mann–Whitney *U* test. Differences were considered to be significant, if p values were <0.05 as assessed by the Mann–Whitney *U* test. The PCA of repertoires of antibody reactivities performed individually for each group of individuals allowed the calculation of respective variances. Variances were compared using the *F* test. For more detailed background information on the multivariate analysis of blot data, we refer to recent literature [10,11,16].

2.4. Analysis of autoantibody binding capacity using biosensor technology

Binding of anti-platelet antibodies to whole platelets and to purified GPIIb/IIIa was analysed under real-time conditions using optical biosensor technology (IAsys platform, Thermo Labsystems, Helsinki, Finland). The IAsys platform analyses biomolecular interactions using an optical biosensor based on resonant mirror technology. The read out parameter of an interaction is the intensity of resonated light during the interaction process, expressed as units of arc seconds (arc s), a measure of the resonant angle. For biosensor experiments, we used exclusively disease-associated autoantibodies that had been eluted from the surface of patients' platelets. Eluates regarded to be appropiate for this series of experiments contained either exclusively immunoglobulin of isotype IgM or of isotype IgG, as verified by ELISA. Human *normal* platelets, adjusted to an concentration of 5×10^5 platelets/μl in PBS pH 7.4, purified platelet membrane GPIIb/IIIa obtained from Calbiochem® (EMD Biosciences, Inc. La Jolla, CA, USA; 100 μg/ml in PBS pH 7.4), and BSA (Sigma, St. Louis, USA; 1 mg/ml in PBS pH 7.4) were immobilized to planar carboxylate cuvettes according to protocols recommended by the manufacturer. In brief, EDC-NHS chemistry was used to link ligands via NH_2 groups covalently to the surface, followed by blocking of unreacted sites with 0.1 M ethanolamine. Immobilisation of 2.36 ng/mm^2 of platelets, 1.22 ng/mm^2 of GPIIb/IIIa and 1.07 ng/mm^2 of BSA was achieved. Binding assays were performed in HBS running buffer pH 7.4 (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA); a part of the assays was additionally performed in PBS running buffer pH 7.4 to exclude negative results due to disruption of conformational epitopes of GPIIb/IIIa in the presence of EDTA. After baseline adjustment, binding partners, i.e. different antibody preparations as well as BSA, adjusted to concentrations ranging from 500 to 1 ng/ml, were allowed to associate with immobilised ligands for 5 min. Dissociation was followed for 10 min. The surface was regenerated with 0.1 M HCl for 3 min. Binding experiments were performed as triplicates. Data were analysed using the manufacturer's software for IAsys instruments and the StatView software.

3. Results

3.1. Natural autoantibody repertoires of AITP patients differ in dependence of the isotype of disease-associated autoantibodies

Multiparametric statistical analysis clearly discriminated self-reactive antibody repertoires of IgM in plasma, IgG in plasma, and IgG purified from plasma of patients with

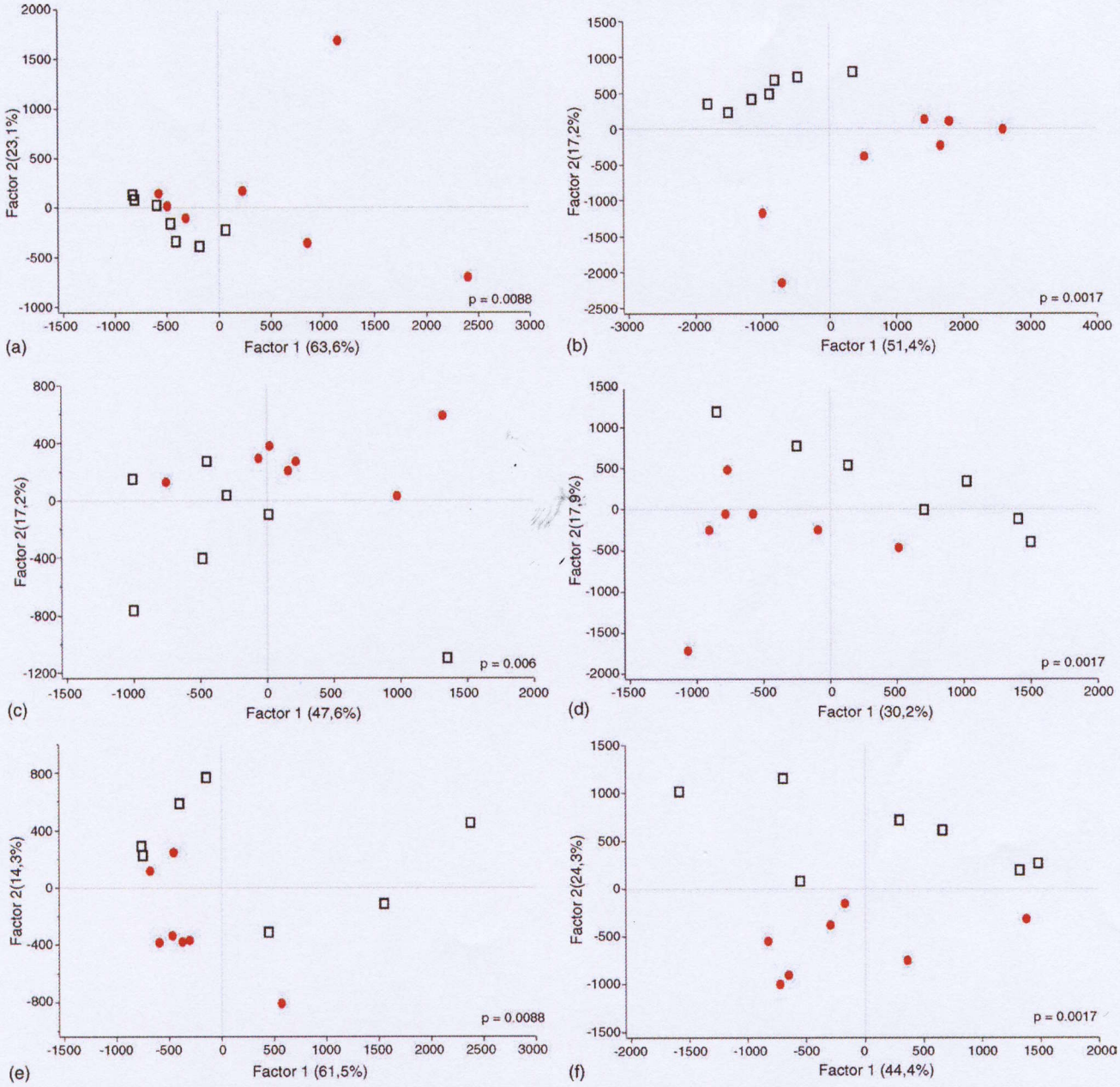

Fig. 1. Multiparametric statistical analysis of self-reactive antibody repertoires of patients with IgM-mediated AITP and of patients with IgG-mediated AITP. IgM and IgG in plasma and IgG purified from plasma of patients with IgM-mediated AITP and of patients with IgG-mediated AITP were analysed by immunoblotting on panels of self-antigens. Densitometric profiles of reactivity of antibodies of individuals were divided into sections corresponding to the individual peaks of immunoreactivity in each profile. The respective areas under the curve were calculated for each peak of reactivity for each individual and submitted to principal component analysis (PCA). PCA data were fitted within a two-dimensional linear subspace (factor 1/factor 2). Depicted is the result of the PCA of the repertoires of reactivities of immunoglobulins towards antigens in platelet extracts (PLT) and of the cumulative PCA of the repertoires of reactivities of immunoglobulins towards the antigens in liver, kidney and erythrocyte extracts (non-PLT). Percentages of variance accounted for by factor 1 and factor 2 are indicated on the abscissa and ordinate, respectively. Each symbol represents the reactivity of immunoglobulin of a single individual for the group of patients with IgM-mediated AITP (filled circle) and for the group of patients with IgG-mediated AITP (open square). The level of statistical significance of discrimination between repertoires of groups of individuals was investigated by linear discriminant analysis (LDA) on the PCA data and by comparing LDA data by means of a Mann–Whitney *U* test. (a) antibody repertoire of IgM, PLT; (b) antibody repertoire of IgM, non-PLT; (c) antibody repertoire of IgG in plasma, PLT; (d) antibody repertoire of IgG in plasma, non-PLT; (e) antibody repertoire of IgG purified from plasma, PLT; and (f) antibody repertoire of IgG purified from plasma, non-PLT.

Table 1
Discrimination of self-reactive antibody repertoires by multiparametric statistical analysis

PCA/LDA	Antibody repertoire of IgM	Antibody repertoire of IgG in plasma	Antibody repertoire of IgG purified from plasma
IgM-mediated AITP vs. IgG-mediated AITP, independent of glycoprotein specificity			
PLT	**0.0088**	**0.006**	**0.0088**
Non-PLT	**0.0017**	**0.0017**	**0.0017**
Anti-GPIIb/IIIa- vs. non-anti-GPIIb/IIIa-mediated AITP, independent of isotype			
PLT	0.1556	0.6985	0.2453
Non-PLT	0.0528	0.7963	0.5186
Anti-GPIIb/IIIa-mediated AITP, isotype IgM vs. IgG			
PLT	**0.0253**	**0.0253**	**0.0253**
Non-PLT	**0.0253**	**0.0253**	**0.0253**
Non-anti-GPIIb/IIIa-mediated AITP, isotype IgM vs. IgG			
PLT	**0.0495**	**0.0495**	**0.0495**
Non-PLT	**0.0495**	**0.0495**	**0.0495**

The table depicts the level of statistical significance of discrimination of self-reactive antibody repertoires of IgM and IgG in whole plasma and of IgG purified from plasma between groups of individuals. Given are p values, derived from submitting PCA data to LDA. Factors 1 of the LDA generated from each immunoreactivity profile were compared by means of a Mann–Whitney U test. Differences were considered to be statistically significant, if p values were <0.05. Antibody repertoires that were significantly different between groups of individuals are depicted in bold numbers. vs., versus; PLT, PCA performed on immunoreactivity profiles analysed on extracts of platelets; non-PLT, cumulative PCA, performed on immunoreactivity profiles analysed on extracts of liver, kidney and erythrocytes.

IgM-mediated AITP ($n=7$) and patients with IgG-mediated AITP ($n=7$) not only in case of extracts of platelet antigens ($0.006<p<0.0088$), but also in the case of non-platelet antigens (liver, kidney, and erythrocytes; $p=0.0017$) (Fig. 1, Table 1). Groups of individuals were homogeneous (Table 2), indicating that differences apart from differences in antibody repertoires that might exist between the group of patients with IgM-mediated AITP and the group of patients with IgG-mediated AITP do not contribute to the observed differences between antibody repertoires.

3.2. *Natural autoantibody repertoires of AITP patients do not differ in dependence of glycoprotein specificity of disease-associated autoantibodies*

Comparative analysis of self-reactive antibody repertoires of patients with anti-GPIIb/IIIa-mediated AITP ($n=8$) and of patients with non-anti-GPIIb/IIIa mediated AITP ($n=6$), regardless of autoantibody isotype, revealed similar antibody repertoires of IgM in plasma, IgG in plasma and IgG purified from plasma toward platelet antigens and toward non-platelet antigens ($0.1556<p<0.6985$ in case of platelet antigens/$0.0528<p<0.7963$ in case of non-platelet antigens, Table 1 and Fig. 2a). Groups of patients were homogeneous as indicated by the F test (Table 2). However, patients with anti-GPIIb/IIIa-mediated AITP and patients with non-anti-GPIIb/IIIa mediated AITP could be subdivided at the basis of autoantibody isotype ($p<0.05$). Although the statistical analysis of differences by LDA and Mann–Whitney U test (Table 1) has to be interpreted with the provision that only small numbers of individuals were available for analysis (five versus three and three versus three), PCA graphs clearly discriminate the two groups (Fig. 2b and c). The difference of immunoreactivities of IgG in plasma toward non-platelet

Table 2
Quotient of total variances of self-reactive antibody repertoires of groups of individuals

Variances	Antibody repertoire of IgM	Antibody repertoire of IgG in plasma	Antibody repertoire of IgG purified from plasma
IgM-mediated AITP vs. IgG-mediated AITP			
PLT	2.14	1.14	2.7
Non-PLT	3.87	1.37	1.44
Anti-GPIIb/IIIa- vs. non-anti-GPIIb/IIIa-mediated AITP, independent of isotype			
PLT	1.65	1.31	1.71
Non-PLT	1.50	1.47	1.27
Anti-GPIIb/IIIa-mediated AITP, isoytpe IgM vs. IgG			
PLT	1.05	1.22	1.04
Non-PLT	1.97	**9.56**	1.09
Non-anti-GPIIb/IIIa-mediated AITP, isotype IgM vs. IgG			
PLT	2.65	4.70	1.41
Non-PLT	1.34	1.66	1.50

Total variances of repertoires of antibody reactivities of IgM and IgG in whole plasma and of IgG purified from plasma with self-antigens were calculated separately in a 41–64-dimension vector space, depending on the tissue extract, for the different groups of individuals. Variances were compared using the F test. Significant differences between total variances are depicted in bold number.

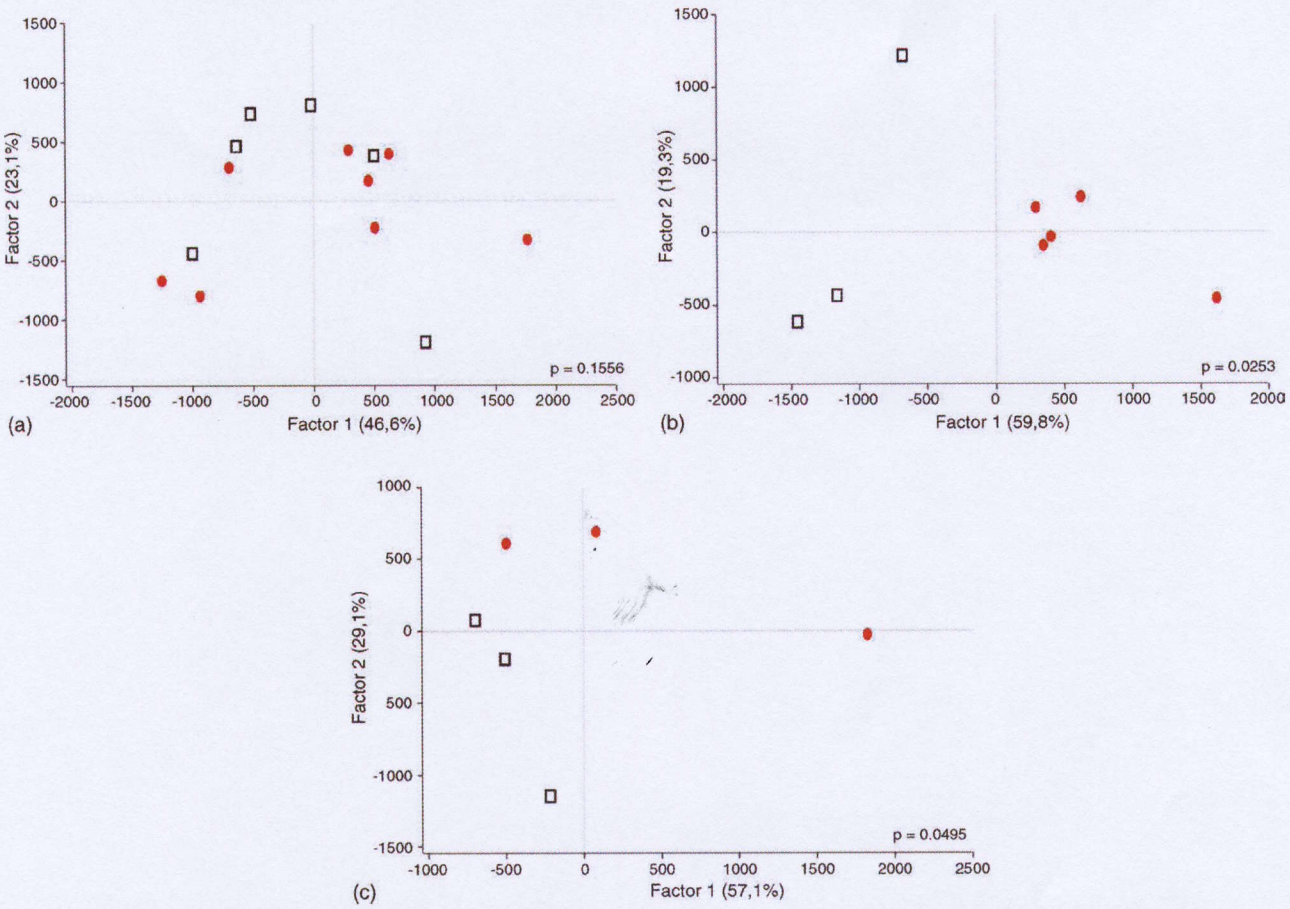

Fig. 2. Multiparametric statistical analysis of self-reactive antibody repertoires of patients with AITP in dependence of isotype and glycoprotein specificity. The analysis was performed as described in the legend to Fig. 1. (a) anti-GPIIb/IIIa mediated AITP vs. non-anti-GPIIb/IIIa mediated AITP, independent of antibody isotype, antibody repertoire of IgM, PLT; (b) anti-GPIIb/IIIa mediated AITP, isotype IgM, vs. isotype IgG, antibody repertoire of IgM, PLT; and (c) non-anti-GPIIb/IIIa mediated AITP, isotype IgM, vs. isotype IgG, antibody repertoire of IgM, PLT.

extracts between anti-GPIIb/IIIa mediated AITP of isotype IgM and anti-GPIIb/IIIa mediated AITP of isotype IgG might be influenced by differences between patients' characteristics apart from antibody repertoires, but not differences of immunoreactivities of IgM in plasma and IgG purified from plasma (Table 2).

3.3. Comparison of immunoglobulin binding capacity in dependence of immunoglobulin isotype and glycoprotein specificity using biosensor technology

We analysed binding of IgM in eluates derived from platelets of patients with anti-GPIIb/IIIa mediated AITP ($n=3$), IgG in eluates derived from platelets of patients with anti-GPIIb/IIIa mediated AITP ($n=1$), IgM in eluates derived from platelets of patients with non-anti-GPIIb/IIIa mediated AITP ($n=3$), and IgG in eluates derived from platelets of patients with non-anti-GPIIb/IIIa mediated AITP ($n=2$). Immunoglobulin concentrations were adjusted to 20 ng/ml in the case of IgM antibodies, and to 100 ng/ml in the case of IgG antibodies in order to adjust for the number of antigen binding sites. When analysed toward whole platelets, binding intensity of immunoglobulins differed in dependence of autoantibody isotype, but not in dependence of autoantibody glycoprotein specificity (Fig. 3a). The difference in binding intensity between antibodies of isotype IgM, regardless of antibody specificity ($n=6$), and antibodies of isotype IgG, regardless of antibody specificity ($n=4$), was statistically significant ($p=0.01$, Mann–Whitney U test). Binding to whole platelets was related to specific binding of anti-platelet autoantibodies, as indicated by the observation that BSA (100 ng/ml) did not show any binding at all (data not shown), and that IgM and IgG, adjusted to 100 ng/ml in both of the cases, in plasma of patients only exhibited around 10% of the binding intensity that we observed with immunoglobulins in eluates (Fig. 3b). In contrast to binding capacity of autoantibodies to whole platelets, binding capacity of autoantibodies to purified GPIIb/IIIa showed dependence on glycoprotein specificity (Fig. 3c).

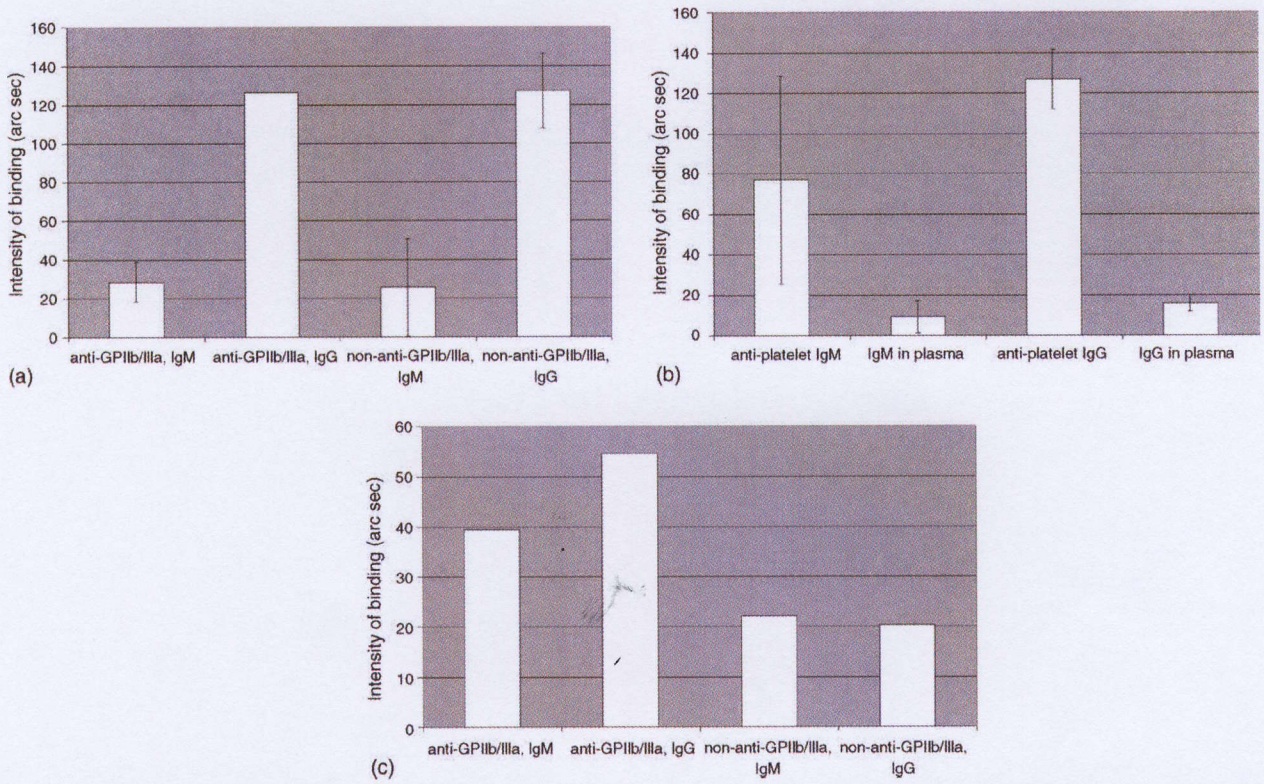

Fig. 3. Analysis of autoantibody binding using biosensor technology: (a) shows binding of anti-platelet autoantibodies in eluates obtained from patients' platelets to whole platelets (IgM 20 ng/ml, IgG 100 ng/ml). Data represent the mean values and the standard deviation of binding intensity of antibodies of three patients in the case of anti-GPIIb/IIIa, isotype IgM, three patients in the case of non-anti-GPIIb/IIIa, isotype IgM, and two patients in the case of non-anti-GPIIb/IIIa, isotype IgG. Only one patient with anti-GPIIb/IIIa, isotype IgG, was available for this experiment; (b) demonstrates the specificity of binding of anti-platelet autoantibodies to platelets by comparing the intensity of binding of both, IgM in eluates from the platelet surface vs. IgM in plasma, and IgG in eluates from the platelet surface vs. IgG in plasma (IgM 100 ng/ml; IgG 100 ng/ml). The graph gives the mean values and the standard deviation of binding intensity of antibodies of six patients in the case of anti-platelet IgM, of four patients in the case of plasma IgM, of four patients in the case of anti-platelet IgG, and of four patients in the case of plasma IgG; and (c) depicts binding of anti-platelet autoantibodies in eluates obtained from patients' platelets to GPIIb/IIIa purified from human platelet membranes (IgM 10 ng/ml, IgG 50 ng/ml). Each binding experiment has been performed with antibodies of a single patient.

4. Discussion

Current understanding of immune network interactions mediated by immunoglobulins focuses on the role of idiotypes expressed on antibody variable regions. Idiotype interactions account significantly for the functional integrity of natural self-reactive antibody repertoires, whereas immunoglobulin isotypes are not considered to direct natural autoimmunity. Using an experimental and computational approach that overcomes the limitations of investigating isolated components of a complex biological system, we here provide evidence of a link between isotypes of disease-associated autoantibodies and the expression of natural autoantibody repertoires in humans.

The methodological approach of quantitative immunoblotting followed by multiparametric statistical analysis of the data is a technique allowing to depict the complex nature of interactions between complex antibody mixtures such as human plasma toward complex antigen mixtures extracted from human cells and tissues and its functional dynamics. The power of multiparametric analysis of profiles of immunoglobulin reactivities with self-antigens by principal component analysis and linear discriminant analysis lies in the reliable discrimination of groups of individuals while minimizing the influence of individual differences on the outcome of the analysis [10,21]. Originally developed in mice [22,23], the technique has been adapted to the analysis of human antibody repertoires, and sufficient data obtained by this technique are available helping to assess the validity of results in the light of existing knowledge in physiology and disease [1,11]. However, quantitative immunoblotting still has the disadvantage of investigating the reactivity of native immunoglobulin toward at least partially denatured antigen. We therefore used biomolecular interaction analysis under real time conditions to verify the significance of autoantibody isotype in relation to autoantibody glycoprotein specificity for the immunoglobulin – self-antigen interaction. Binding assays of anti-platelet autoantibodies using biosensor technology revealed that binding intensity of autoantibodies to whole platelets, i.e. to self-antigen in its physiological native form, does not depend primarily on antibody glycoprotein specificity but on immunoglobulin isotype, consistent with

the rules of affinity selection of antibodies and isotype switch, whereas binding intensity to purified GPIIb/IIIa, i.e. self-antigen in a non-physiological form, is influenced primarily by glycoprotein specificity. Thus, although detailed knowledge on autoantibody fine-epitope specificity is undoubtedly of central importance for investigations aiming at interfering with mechanisms of antigen-presentation at the single cell level, our data indicate that under the point of view of complex in vivo interactions of cell populations immunoglobulin isotype is of higher relevance than glycoprotein specificity for the final outcome of the interaction.

The binding capacity of self-reactive IgG in normal human plasma is controlled by several molecular interactions including the interaction with anti-idiotypic IgG and IgM [24,25]. Immunoglobulin interactions that fine tune the reactivity of IgG autoantibodies in plasma may be altered in pathological conditions [16]. In the current study, we observed that the isotype of disease-associated autoantibodies influenced selection of natural self-reactive antibody repertoires of IgM and IgG in plasma as well as natural self-reactive antibody repertoires of IgG purified from plasma, indicating that altered antibody repertoires of IgG in plasma of AITP patients are not due to altered peripheral control mechanisms, but due to alterations of the self-reactive antibody repertoire of the IgG isotype as such. Isotype-dependent alterations of natural self-reactive antibody repertoires of AITP patients were not influenced by differences between patients' characteristics apart from antibody repertoires, as indicated by the analysis of variances of the PCA of repertoires of antibody reactivities performed individually for each group of individuals, with one exception: The difference of immunoreactivities of IgG in plasma toward non-platelet extracts between anti-GPIIb/IIIa mediated AITP of isotype IgM and anti-GPIIb/IIIa mediated AITP of isotype IgG might be influenced by differences between patients' characteristics apart from antibody repertoires. However, differences of immunoreactivities of IgG purified from plasma toward non-platelet extracts between anti-GPIIb/IIIa mediated AITP of isotype IgM and anti-GPIIb/IIIa mediated AITP of isotype IgG were *not* influenced by differences between patients' characteristics apart from antibody repertoires, as indicated by the *F* test, supporting our conclusion that the self-reactive antibody repertoire of the IgG isotype as such is altered by isotypes of disease associated autoantibodies even when comparing the subgroups of patients with anti-GPIIb/IIIa mediated AITP of isotype IgM and anti-GPIIb/IIIa mediated AITP of isotype IgG.

Taken together, isotype differences of disease-associated autoantibodies are associated with altered natural self-reactive antibody repertoires in humans. Our data suggest that – at least under certain conditions – regulation of natural autoreactivity by antibody isotype may occur. Alternatively, different pre-existing repertoire structures might have influenced the isotype preference in individual patients' platelet-bound antibodies. Based on the following considerations, we do not consider the latter hypothesis to sufficiently explain our observations:

(i) The natural self-reactive antibody repertoires of healthy individuals have been intensively investigated in the past. The results provided evidence that natural self-reactive antibody repertoires of healthy individuals are restricted to a limited subset of immunodominant autoantigens that is selected early in development, and remains conserved between individuals through aging [18,19,26–28]. Thus, pre-existing repertoire structures in *healthy individuals* are similar.

(ii) The calculation of the total variances of repertoires of antibody reactivities of IgM and IgG in whole plasma and of IgG purified from plasma with self-antigens for the different groups of individuals, followed by the comparison of variances by the *F* test, did not provide evidence for heterogeneity between the group of patients with IgM-mediated AITP and patients with IgG-mediated AITP. Thus, the statistical approach does not argue in favour of specific differences such as different pre-existing repertoire structures in patients with IgM-mediated AITP and patients with IgG-mediated AITP that might predispose to the development of either IgM-mediated AITP or IgG-mediated AITP.

(iii) Experimental data indicate that natural immunoglobulins underly patterns of temporal changes in their circulating levels, and that the appearance of disease-associated autoantibodies is principally linked to the disturbance of normal patterns of natural autoantibody repertoire dynamics in mice as well as in patients exhibiting autoimmune disease [29]. Such disturbance of normal patterns of natural autoantibody repertoire dynamics has been observed in systemic autoimmune disease and in organ-specific autoimmune disease. The discussion whether the disturbance of normal patterns of natural autoantibody repertoires is the cause or the consequence of disease-associated autoantibodies might be regarded to be open in the case of *systemic* autoimmune diseases. In the case of *organ-specific* autoimmune diseases, however, the typical oligoclonality of disease-specific autoantibodies favours the view that alterations of natural antibody repertoires follow the expansion of a few B cell clones specific for a well defined target antigen. There is no obvious reason why a pre-existing broadly disturbed natural autoantibody repertoire structure should foster selectively the expansion of a few organ-specific B cell clones. AITP is an organ-specific autoimmune disease, and the oligoclonality of disease-associated autoantibodies in AITP has been demonstrated [13].

Thus, taking into consideration regulation of natural autoreactivity by antibody isotype on the background of established knowledge on the role of immunoglobulin *idiotypes* for B cell selection [7–9], our observations suggest that natural self-reactivity might be determined by a hierarchical order of interactions of immunoglobulin structures with B lymphocytes: Antigen fine epitope specificity, i.e. the

idiotype, regulates the selection of self-reactive B cells at the clonal level. The immunoglobulin isotype however might direct the selection of self-reactive B cell *repertoires* at the supraclonal level. Therefore, an altered isotype balance of the overall immunoglobulin pool might directly contribute to the antigenic composition as well as to the degree of activity of self-reactive B cells, and determine the finally active natural self-reactive B cell repertoire. The underlying mechanisms of isotype-mediated selection of natural antibody-repertoires might target directly or indirectly the immunoglobulin–BCR interaction, as it has been shown for isotype-mediated selection of disease-associated autoantibody repertoires [30–32] and in experimental models [33–35], or influence the degree of activity of the entire B cell repertoire via the interaction of immunoglobulins with B cell surface structures except from the BCR such as complement receptors CR1 (CD35) and CR2 (CD21) [36,37], FcγRIIb with subsequent feedback inhibition of antibody production [38], or a recently described Fc receptor for IgM, constitutively expressed by the majority of B lymphocytes [39]. The concept of an isotype-specific selection of natural antibody-repertoires is principally in line with recently published work on the IgE immune response toward allergens, indicating that the immunoglobulin isotype of the antibody recognizing an allergen directs somatic diversity of the immunoglobulin repertoire [40].

Previous data in mice and in men indicated that organ-specific, clonally restricted *disease*-associated autoantibodies are related somehow to non-organ specific broad alterations of *natural* self-reactive antibody repertoires [16,29]. The structural basis for such synchronization of autoantibody dynamics has not been clarified yet. Control of natural self-reactive antibody repertoires by immunoglobulin isotypes at a supraclonal level would be able to explain the synchronization of dynamics of disease-associated and natural autoantibodies at a structural level. Our data thereby may provide new insights into the boundaries between physiological autoimmunity and pathological autoaggression.

Taken together, the significance of immunoglobulin isotypes for the regulation of natural autoreactivity seems to be currently underestimated: Our data suggest that regulation of natural autoreactivity by antibody isotype might occur under certain conditions. Taking into account control of natural self-reactive antibody repertoires by immunoglobulin isotypes at a supraclonal level may provide new insights into functional mechanisms of immunoglobulin-dependent immunoregulation in humans, and may help to define new strategies to influence autoimmune responses.

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