Journal of Immunological Methods 403 (2014) 26–36

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Non-specific binding in solid phase immunoassays for autoantibodies correlates with inflammation markers

Esin Güven [a], Karen Duus [a], Magnus Christian Lydolph [a], Charlotte Sværke Jørgensen [b], Inga Laursen [a,1], Gunnar Houen [a,*]

[a] Department of Clinical Biochemistry, Immunology and Genetics, Statens Serum Institut, Copenhagen, Denmark
[b] Department of Microbiological Diagnostics & Virology, Statens Serum Institut, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 26 August 2013
Accepted 19 November 2013
Available online 25 November 2013

Keywords:
ELISA
Autoantibodies
Non-specific binding
Elevated immunoglobulin concentration
Inflammation

ABSTRACT

Enzyme-linked immunosorbent assay (ELISA) is a validated and sensitive method for detection of human autoantibodies, but may have problems with specificity. Non-specific binding is a well-known problem often observed in tests for autoantibodies, when sera are incubated on plastic surfaces, e.g. an ELISA plate. To understand the mechanisms underlying non-specific immunoglobulin deposition, we here analyse the phenomenon in detail and we propose means of reducing false positive test results caused by non-specific binding.

The level of non-specific binding, in sera with suspected autoreactivity, was analysed in non-coated and autoantigen-coated ELISA wells and 4–32% of sera showed a high level of non-specific binding depending on the assay conditions and serum properties. Non-specifically binding sera were found to contain increased concentrations of IgG and other inflammatory mediators. Moreover, non-specific binding could be induced in serum by increasing the concentration of IgG and incubating the serum at 40 °C. This suggests that non-specific binding immunoglobulins can be formed during inflammation with high immunoglobulin levels and elevated temperature. We show that the level of non-specific binding correlates with the IgG concentration and therefore propose that non-specific binding may be interpreted as an informative finding indicative of elevated IgG and inflammation.

1. Introduction

1.1. Autoantibody-based diagnostics and ELISA

Both cellular and humoral immunity can be involved in autoimmune diseases and autoantibodies found in body fluids can be used as a tool for correct diagnosis (Voller et al., 1976). If autoimmunity is suspected, patient sera should be screened for autoantibodies, for example using indirect immunofluorescence, determining reactivity towards antigens in the used tissue/cell preparation. If a vasculitis disease is suspected, antibodies directed against neutrophil granules are traditionally screened for by indirect immunofluorescence and quantified using ELISA plates coated with the neutrophil proteins myeloperoxidase (MPO) and proteinase 3 (PR3) (Cohen Tervaert and Damoiseaux, 2012; Schultz and Tozman, 1995).

Abbreviations: AP, alkaline phosphatase; AUFS, absorbance units full scale; BSA, bovine serum albumin; CRP, C-reactive protein; C3, complement component 3; ELISA, enzyme-linked immunosorbent assay; β2GP1, β2 glycoprotein I; ICs, immune complexes; IVIg, intravenous immunoglobulin; MPO, myeloperoxidase; NHS, normal human serum; *p*NPP, *para*-nitrophenylphosphate; PR3, proteinase 3; RFs, rheumatoid factors.

* Corresponding author at: Department of Clinical Biochemistry, Immunology and Genetics, Artillerivej 5, Statens Serum Institut, 2300 Copenhagen, Denmark. Tel.: +45 32688241; fax: +45 32683876.
E-mail address: gh@ssi.dk (G. Houen).

[1] Deceased 11.12.2011.

http://dx.doi.org/10.1016/j.jim.2013.11.014
0022-1759

If systemic lupus erythematosus or Sjögren syndrome is suspected, antibodies to Ro52, Ro60 and La are of diagnostic value in addition to DNA antibodies (Hernandez-Molina et al., 2011; Moutsopoulos and Zerva, 1990). If anti-phospholipid syndrome is under consideration, cardiolipin or more specifically β2 glycoprotein I (β2GP1) antibodies should be determined (Shovman et al., 2007; Meroni et al., 2011).

1.2. ELISA and non-specific binding

ELISA is a high throughput, practical method and has been used for several decades for analysis of human antibodies (Engvall and Perlmann, 1971; van Weemen and Schuurs, 1971). However, ELISA may have problems with specificity (Reen, 1994; Butler, 2000), and non-specific deposition of immunoglobulins can lead to false positive results and ultimately a wrong diagnosis and treatment.

False positive results can be caused by different phenomena. These include inadequate blocking, where immunoglobulins may adsorb directly to the polystyrene surface, and binding of antibodies to the blocking agent(s) (Jørgensen et al., 2005; Peterfi and Kocsis, 2000; Xiao and Isaacs, 2012). Bovine serum albumin (BSA) and milk proteins are routinely used as blocking agents and antibodies directed against these proteins may cause false positive results (Andersen et al., 2004; Willman et al., 1999). Non-specific binding has also been ascribed to heterophile antibodies and anti-animal antibodies have been described to interfere with specific ELISA systems (Kaplan and Levinson, 1999; Levinson, 1992; Levinson and Miller, 2002; Kricka, 1999). In capture ELISAs rheumatoid factors (RFs) may pose a major problem since they can bind antibodies and thereby obstruct the detection of specific antibodies (Lewis et al., 2003). Also, antibodies against complement components may cause false positive results, as the polystyrene surface activates the complement system, generating neoisotopes targeted by antibodies as seen for C3 nephritis factor (Andersson et al., 2002; Nilsson et al., 1993). In a similar manner immune complexes (ICs) may be drawn to the surface, resulting in false positivity (Kricka, 1999; Hu et al., 1992; McFarlane et al., 1990).

1.3. Aim of this study

In this study, we evaluated the occurrence of non-specific binding of sera in the autoantibody ELISAs mentioned above, characterised the phenomenon of non-specific deposition of immunoglobulins and evaluated the different steps of the ELISAs to reduce the level of non-specific binding.

2. Materials and methods

2.1. Chemicals and buffers

Alkaline phosphatase (AP) substrate buffer (1 M ethanolamine, 0.5 mM $MgCl_2$, pH 9.8), incubation buffer (0.05 M Tris, 0.15 M NaCl, 0.2% BSA, 0.05% polysorbate 20, 0.02% sodium azide, 0.001% phenole red, pH 7.5), washing buffer (0.154 M NaCl, 10 mM Tris, 0.005% Tween 20, pH 7.5), and carbonate buffer (0.05 M sodium carbonate, pH 9.6). IVIg, calf serum and rabbit serum were from Statens Serum Institut (Copenhagen, Denmark). BSA, ovalbumin, AP-conjugated secondary antibodies (goat anti-human IgG, IgM, IgA), protein G and AP-substrate tablets were from Sigma-Aldrich (St. Louis, Missouri, USA). Skimmed milk powder and polysorbate 20 (tween 20) were from Merck (Darmstad, Germany).

2.2. Sera

Control sera were obtained from healthy volunteers and used anonymously. Normal human serum (NHS) was used as a pool of 10 sera.

Sera observed to exhibit non-specific binding in MPO/PR3/Ro/La/β2GP1 autoantibodies analyses were obtained from the routine diagnostic laboratories at the Department of Clinical Biochemistry, Immunology and Genetics, Statens Serum Institut. Altogether, 847 sera submitted for 1707 autoantibody tests were used. In Table 1, relative non-specific binding sera (sticky sera) were defined as sera where IgG deposition on a non-coated ELISA well exceeded the cut-off level for the analysis (>10 units/ml). For other tables and figures absolute non-specific binding was defined as absorbance readings above 0.2 absorbance units full scale (AUFS) (A405/650), with NHS values below 0.2 AUFS (A405/650). Sera with a positive serologic profile for bacterial antibodies were obtained from the Department of Microbiological Diagnostics and Virology, Statens Serum Institut. Selection criteria were a positive antibody response for the indicated bacteria.

2.3. IC assay

Sera were analysed for ICs using CirculatingIC-C1q MicroVue™ kit from Quidel (Sissach, Switzerland), following the recommendations from the manufacturer.

2.4. Preparation of heat-induced IC

ICs were made by heating IVIg for 10 min at 56 or 60 °C. In some experiments, IVIg was heated for different times at different temperatures to investigate the time and temperature profile for induction of non-specifically binding Igs.

2.5. ELISA

Maxisorp microtitre plates were incubated with carbonate buffer overnight at 2–6 °C or 2 h at room temperature. Blocking of residual binding sites was performed using washing buffer for 30 min at room temperature. Antibodies against human IgG, IgM or IgA were diluted to 1:5000 in incubation buffer and added in duplicates and incubated for 1 h at room temperature. Following 3× washing, the plate was incubated with AP-conjugated secondary antibody diluted 2000-fold in incubation buffer. After incubation for 1 h and extensive washing, bound antibodies were quantified by addition of *p*NPP (1 mg/ml) dissolved in AP substrate buffer. The colour development was measured after incubation at room temperature for 30 min at 405 nm with background subtraction at 650 nm as reference. Data are presented as mean values of double determinations with error bars indicating the standard deviation.

Table 1
Non-specific immunoglobulin deposition. 847 sera submitted for autoantibody tests (1707 tests in all) in the routine laboratory at Statens Serum Institut were analysed for non-specific immunoglobulin binding to non-coated ELISA wells. Results are presented as number of serum samples and percent (of total serum samples or non-specific binding sera). Non-specific binding sera were defined as sera with immunoglobulin deposition on a non-coated, blocked well above cut-off.

Antigen	Total number of analyses	Positive for antigen	Number of sera with non-specific binding (% of total)	Antigen positive sera of non-specific binders (% of non-specific binders)	False positives (% of total)
MPO (IgG)	184	86	10 (5.4%)	3 (30.0%)	7 (3.8)
PR3 (IgG)	309	119	11 (3.6%)	3 (27.3%)	8 (2.6)
Ro52 (IgG)	322	70	35 (10.9%)	3 (8.6%)	32 (9.9)
Ro60 (IgG)	322	83	91 (28.3%)	8 (8.8%)	83 (25.8)
La (IgG)	322	20	12 (3.7%)	1 (8.3%)	11 (3.4)
$\beta_2$gp1 (IgG)	124	36	40 (32.3%)	4 (10.0%)	36 (29.0)
$\beta_2$gp1 (IgM)	124	33	33 (26.6%)	5 (15.2%)	28 (22.6)
Total	1707	447	232 (13.6%)	27 (11.6%)	205 (12.0)

2.6. Diagnostic ELISA

The antigen of interest was coated on a polystyrene surface (Maxisorp) overnight in Phosphate-Buffered Saline (β2GP1), 7 M urea (Ro52, Ro60, La), carbonate buffer (MPO) or carbonate buffer with 0.01% Triton X-100 (PR3). After coating, the ELISA wells were washed 3 times prior to addition of serum (diluted 1:100 in incubation buffer) for 1 h. After incubation and washing, AP-conjugated goat antibodies directed against human IgG or IgM were allowed to bind for 60 min and quantified using *p*NPP for 30 min. All diagnostic ELISAs used standard sera from which cut-off levels and units were calculated. In Table 1 relative non-specific binding is defined as absorbance values of a non-coated well above the cut-off. To allow comparison of the sera, absolute nonspecific binding was defined as absorbance values above 0.2 AUFS (A405-650) after 30 min of incubation with *p*NPP.

2.7. Detection of complement component 3 (C3) nephritic factor

Analyses for C3 nephritic factor were performed by Labmedicin Skåne, Klinisk immunologi och transfusionsmedicin, enheden för immunologi (Lund, Sweden).

2.8. Quantification of C3 and immunoglobulins

The concentration of C3 and immunoglobulins was measured by rate nephelometry on an Immage instrument (Beckman Coulter, Fullerton, USA) using kits from Beckman Coulter, Fullerton, USA and following the instructions of the manufacturer.

2.9. Quantification of C-reactive protein (CRP)

The serum concentration of CRP was analysed using Time-Resolved Amplified.

Cryptate Emission (TRACE) technology on a Kryptor (BRAHMS AG, Hennigsdorf, Germany) according to the instructions from the manufacturer.

2.10. Statistical analysis

Student's *t*-test was performed using Graphpad Prism (La Jolla, CA, USA).

3. Results

3.1. Non-specific binding in solid phase autoantibody assays

In routine testing for PR3/MPO antibodies it was consistently observed that some sera tested positive for both PR3 and MPO antibodies, while being negative in immunofluorescence tests for neutrophil granulocyte antibodies. For this reason, we developed ELISA assays which systematically screen all sera for non-specific binding using antigen-coated and non-coated wells for all samples. Using these assays, we evaluated 309 sera submitted for PR3 antibody testing and 184 sera submitted for MPO antibody testing (Table 1). Among the samples tested for PR3 antibodies, 119 (39%) tested clearly positive, eight samples showed relative non-specific binding (levels of IgG deposition to non-coated ELISA wells above cut-off) and were negative for specific antibodies (3%), three sera (1%) showed relative non-specific binding but were still PR3 antibody-positive after subtraction of relative non-specific binding. Altogether, approximately 4% of the sera exhibited non-specific binding in the PR3 antibody assay and approximately the 3% were false positives. In the MPO antibody assay, 47% of the 184 sera were found to be positive, 98 were negative, seven (4%) were negative with non-specific binding and three sera (2%) with non-specific binding were positive. Collectively, 5% of the serum samples submitted for MPO autoantibody testing were observed to exhibit non-specific binding and 4% were false positives (Table 1).

Of 322 serum samples, which were analysed for Ro52, Ro60 and La autoantibodies, 22%, 26% and 6% were found to be positive for Ro52, Ro60 and La autoantibodies, respectively, and 35 (11%), 91 (28%) and 12 (4%) sera exhibited relative non-specific binding in these assays. Of the 35 non-specific binding sera found in the Ro52 assay, three were positive whereas eight of the 91 non-specific binding sera in the Ro60 assay were autoantibody positive and one of the 12 sera with relative non-specific binding tested positive in the La autoantibody assay (Table 1). Thus, the number of false positive results varied from 3 to 29%.

Non-specific binding was also analysed in a β2GP1 antibody assay where 124 sera were analysed for both IgG and IgM autoantibodies. 27% of these sera were IgM autoantibody positive and 29% were IgG autoantibody positive. Forty (32%) and thirty-three (27%) sera in the

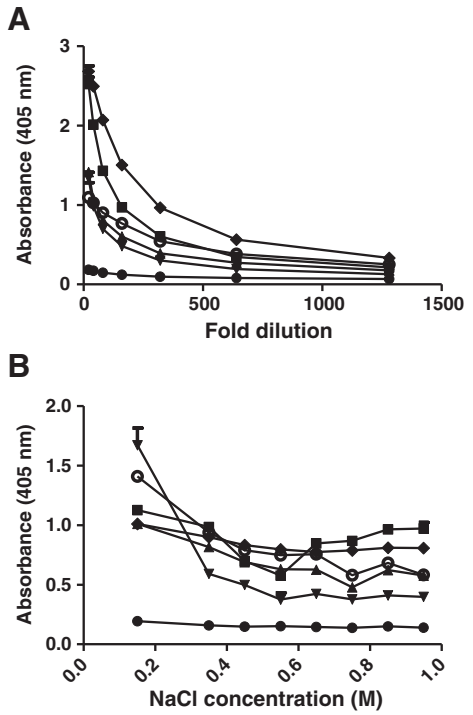

Fig. 1. Characterisation of non-specific binding. A) serum concentration and B) salt dependency of non-specific binding. Five serum samples with high non-specific binding and a control sample (filled black circle) without non-specific binding were incubated in non-coated wells of an ELISA plate using the indicated serum concentration as fold serum dilution (A) or serum diluted 1:100 in Tris buffer containing the indicated concentration of NaCl (B). After incubation at the indicated conditions, deposited IgG was analysed using goat anti-human IgG conjugated to alkaline phosphatase and the substrate *p*NPP.

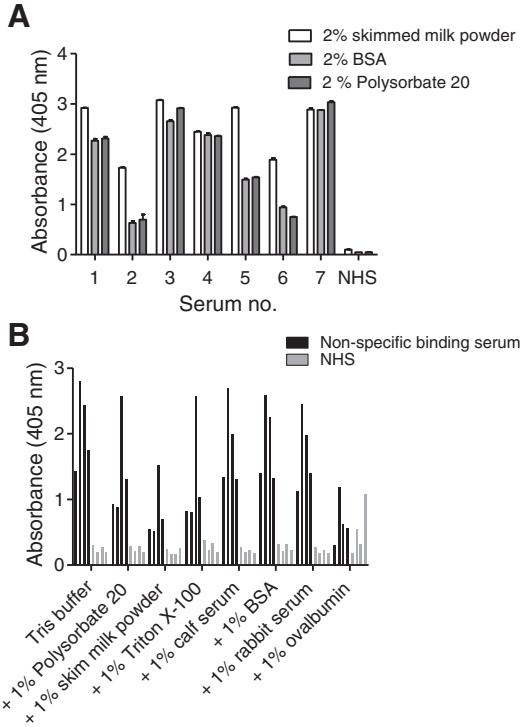

Fig. 2. ELISA conditions to reduce non-specific binding. A) The level of non-specific binding was measured using three different blocking conditions: 2% skimmed milk, 2% bovine serum albumin (BSA), or 2% polysorbate 20 for 30 min using seven sera with non-specific binding and a control serum pool (NHS — normal human serum). B) The level of non-specific binding in eight sera (four with non-specific binding, four without) was measured using a number of different additives to the Tris incubation buffer (1% polysorbate 20, skimmed milk, Triton X-100, calf serum, bovine serum albumin (BSA), rabbit serum and ovalbumin. The following steps of the ELISA procedure were performed as described in Materials and methods.

β2GP1 assay showed relative non-specific binding when analysed for deposition of IgG and IgM, respectively. 10% of the sera showing relative non-specific binding in the β2GP1 IgG assay were autoantibody positive and 15% in the β2GP1 IgM assay with relative non-specific binding were autoantibody positive.

Overall, the extent of relative non-specific binding varied from 4 to 32% of the analysed sera (3–29% false positives) and is thus a significant threat to correct serodiagnostics (Table 1). Due to this significant percentage of sera showing non-specific binding we sought to investigate the cause of antigen-independent immunoglobulin deposition of some sera and to evaluate the ELISA setup to minimize interference with specific autoantibody detection.

3.2. Characterization of non-specific binding

We first wished to characterise the non-specific binding. The dependency on incubation time and concentration (serum dilution) was therefore determined for five non-specific binding sera (Fig. 1A and results not shown). Increasing deposition of IgG was observed with increasing incubation time until 90–120 min, where the binding reached a maximum. The non-specific binding was observed to decrease as a function of serum dilution but with individual dilution curves for the 5 sera presented (Fig. 1A).

The non-specific binding was further investigated by analysing the effect of increasing concentrations of salt and detergent (Fig. 1B and results not shown). The analysed sera were highly sensitive to increasing concentrations of sodium chloride in the incubation buffer and the non-specific binding decreased when the salt concentration was increased. For one serum, the non-specific binding to non-coated wells was reduced with 40%, when the salt concentration was increased from 0.15 M to 0.35 M (Fig. 1B). Increasing the detergent concentration had some inhibitory effect on the non-specific binding, but this effect was highly serum dependent. Only two of the four sera tested were markedly affected (15–30%) in their ability to deposit IgG on a non-coated surface, when the detergent concentration was increased from 0.06 to 1% polysorbate 20 (results not shown).

3.3. Influence of immuno-assay design

Several causes of non-specific binding have previously been proposed. Insufficient blocking could be an obvious problem causing non-specific binding (Peterfi and Kocsis, 2000; Jørgensen et al., 2005; Xiao and Isaacs, 2012), however an increased blocking step (blocking the wells for 1 h with 1% polysorbate 20) was not found to reduce the non-specific

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Absorbance (405 nm)
0.0
0.5
1.0
1.5
2.0
2.5
IgG
IgA
IgM

binding compared with 30 min of blocking (results not shown). Another possible hypothesis of non-specific binding is antibodies directed against blocking proteins. As the blocking solution may contain BSA, skimmed milk or detergent, blocking agent-directed antibodies could cause non-specific binding. Seven samples showing non-specific binding and a control serum pool (NHS) were tested for IgG deposition on a BSA-, skimmed milk-, or polysorbate 20-coated surface (Fig. 2A). In the tested sera, no or only a little increase in non-specific binding was observed on the BSA surface compared to the detergent-blocked surface, suggesting few BSA-directed antibodies whereas increased IgG deposition was found on the skimmed milk surface for five out of seven sera.

We hypothesised that if the polystyrene surface of the ELISA well had the ability to deposit IgG, then preincubation on a polystyrene surface may adsorb the IgG prone for deposition and prevent non-specific binding to an antigen-coated surface. We tested this hypothesis by pre-incubating non-specific binding sera in uncoated ELISA wells for 1 h prior to transfer of the sera to another uncoated, but blocked, well. This pretreatment was found to reduce the non-specific binding, but not to abolish it completely (results not shown).

If non-specific binding antibodies are of low affinity, the presence of competing agents during serum incubation might remove or diminish non-specific binding. We therefore added 1% of the following agents to the 50 mM Tris incubation buffer: BSA, skimmed milk, ovalbumin, rabbit serum, calf serum, polysorbate 20, or Triton X-100, and found that skimmed milk and ovalbumin were good additives to remove non-specific binding but that ovalbumin also induced non-specific binding in some NHS samples (Fig. 2B).

The influence of the conjugate specificity was also investigated, showing that secondary antibodies directed against the whole IgG molecule, gamma chain or kappa chain all detected specific as well as non-specific binding (results not shown).

A majority of autoantibodies with a diagnostic value are of the IgG class and most non-specific binding detected is therefore of IgG class although non-specific IgM deposition can also be observed (as seen in the β2GP1 assay, Table 1). Non-specific binding of the individual Ig classes was investigated in ELISA plates. The immunoglobulin deposition of individual sera was analysed using 16 sera with non-specific IgG deposition. In many of these, non-specific IgM deposition was observed and in several sera, non-specific deposition of IgA could be detected showing that non-specific immunoglobulin deposition often occurs with several isotypes (Fig. 3).

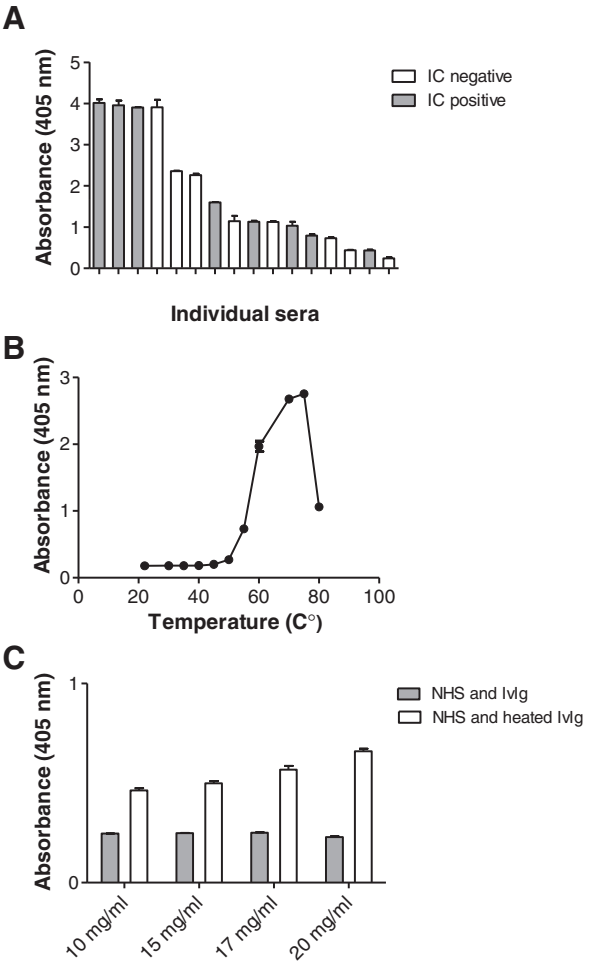

Fig. 4. Non-specific binding can be induced by heating and may be caused by immune-complexes. A) 16 serum samples showing non-specific binding were analysed for the presence of immune complexes (ICs). The level of non-specific binding is depicted on the Y-axis and sera positive for ICs are shaded. B) IVIg (0.6 mg/ml) were heated to the indicated temperatures and added to serum at the indicated concentrations and the level of non-specific binding was analysed. C) Normal human serum (NHS) was supplemented with additional IgG (IVIg) in a concentration of 10, 15, 17, and 20 mg/ml with or without heat treatment (60 °C for 10 min). The level of non-specific binding was analysed on non-coated ELISA wells as described in Materials and methods.

3.4. ICs induce non-specific binding

ICs have been proposed to cause non-specific binding (Hu et al., 1992; Jethwa et al., 2000). The occurrence of ICs in patient sera showing non-specific binding was therefore assessed, which showed that only a proportion of non-specific binding sera contained measurable, C1q-binding ICs (Fig. 4A). Of 16 sera showing non-specific binding, 50% were found to contain circulating ICs, measured by C1q interaction. Circulating ICs are therefore a possible inducer of non-specific binding although a major part of non-specific binding cannot be explained by C1q-binding circulating ICs. No correlation was observed between the level of non-specific binding and measurable ICs.

Artificial, heat-induced ICs were added to normal human serum and shown to induce non-specific binding (Fig. 4B). The induction of immune aggregates was investigated adding IVIg in different concentrations to NHS with or without heating (60 °C, 10 min), showing the induction of non-specific binding to be dependent on heat induction, increasing with increasing

Fig. 3. Subtypes of interfering immunoglobulins. Sixteen sera showing non-specific binding were incubated in non-coated, detergent-blocked ELISA wells and analysed for the type of deposited immunoglobulins using AP-conjugated secondary antibodies specific for human IgG, IgM or IgA. Stippled line represents IgG cut-off (0.2 absorbance units full scale).

immunoglobulin concentration (Fig. 4C). Similar results were obtained with individual sera (results not shown).

3.5. Inflammatory parameters correlate with non-specific binding

In order to further characterise the sera with high non-specific binding in ELISA, we determined immunoglobulin, C3 and CRP concentrations of 11 sera showing non-specific binding (Table 2 and Fig. 5). It was seen that sera with high non-specific binding had higher levels of total IgG compared with NHS pools not showing non-specific binding (Fig. 5A, $P < 0.05$). On the average, total IgG concentrations were 17.9 ± 6.5 mg/ml (mean ± SD) compared to the control group of 10.7 ± 2.3 mg/ml (mean ± SD), and nine of the 11 tested sera with non-specific binding had IgG concentration above the normal range.

Also, IgA levels were higher in five of 11 serum samples showing non-specific binding, whereas IgM concentrations were in the normal range or slightly decreased. All of the 11 sera showing non-specific binding, showed increased immunoglobulin levels of either IgG or IgA type (Table 2).

CRP concentrations were also assessed in the sera showing non-specific binding, with an average CRP concentration that was 10 times higher than the controls (54.9 ± 60.79 compared to 4.7 ± 6.6 mg/l, $P < 0.05$) (Fig. 5B). In conclusion, elevated IgG, elevated IgA, elevated CRP or a combination of these could be observed in all the serum samples with non-specific binding (Table 2).

In order to investigate if increased immunoglobulin levels were the cause of non-specific binding, sera with non-specific binding and increased IgG concentration were diluted to 10 mg IgG/ml and compared with NHS (9.1 ± 1.6 mg/ml IgG) but non-specific binding to a non-coated surface could still be observed in the diluted sera (Fig. 5C), showing that non-specific binding is not solely dependent on immunoglobulin concentration.

3.6. Non-specific binding can occur in sera with recent or on-going infection

We speculated that non-specific binding could be a general trait of inflammation/infection and 70 sera with antibodies to different bacteria were assessed for non-specific deposition of IgG on a non-coated surface (Table 3). The results showed that non-specific IgG deposition could be found in a number of sera with recent or ongoing bacterial infections (Table 3). Non-specific binding was most pronounced in sera positive for antibodies against *Bordetella pertussis*, *Treponema pallidum* and *Salmonella enteritidis/typhimurium*, where 8/10, 7/10 and 5/10 samples showed non-specific binding, respectively. We therefore collected 10 new serum samples with a high positive antibody titre for these bacteria and analysed the level of absolute non-specific binding and the immunoglobulin concentration, confirming that non-specific binding sera had elevated IgG concentrations and showing a correlation between immunoglobulin concentration and level of non-specific binding (Fig. 6). We realized that part of the serum samples had actually been heat treated (56 °C for 30 min, to inactivate complement) prior to analysis for antibodies against *T. pallidum* and prior to detection of non-specific binding. A third collection of 44 new samples without heat treatment was therefore collected and analysed. Only one serum showed intense non-specific binding whereas the 43 serum samples showed little or no non-specific binding (AUFS < 0.3) (Fig. 7). The serum samples were heated at 40 °C overnight or at 56 °C for 30 min showing that non-specific binding was induced (Fig. 7A) and that the level of non-specific binding correlated with serum IgG concentration after heat treatment (Fig. 7B).

Repeated freezing/thawing of sera (−20 °C or −80 °C or −135 °C for 1–10 cycles) did not induce non-specific binding (results not-shown).

3.7. Non-specific binding can be induced in NHS

As elevated immunoglobulin concentrations were found in sera with non-specific binding and since non-specific binding could be induced in NHS by heat treatment, we analysed if non-specific binding could be induced under conditions that approached the physiological conditions found in patients with systemic autoimmune diseases or infections. IVIg was added to NHS (without non-specific

Table 2
Inflammatory markers in non-specific binding sera. 11 sera with non-specific binding in the MPO/PR3 ELISA were analysed for IgG-, IgA- and IgM-concentration, the concentration of C-reactive protein (CRP) and complement component 3 (C3). Non-specific binding was defined as IgG deposition on a blocked, non-coated surface. A * represents low non-specific binding (0.2 < A405 < 0.3), two ** represent medium non-specific binding (0.3 < A405 < 0.5) and three *** represent high non-specific binding (A405 > 0.5). The reference intervals are shown in brackets and values outside the reference interval are shown in bold. Also, 11 control sera without non-specific binding were analysed and the analysed serum components were inside the reference intervals (not shown in the table).

Year of birth	Non-specific binding	IgG (6.4–13.5 g/l)	IgA (0.70–3.12 g/l)	IgM (0.56–3.52 g/l)	CRP (0–10 mg/l)	C3 (0.79–1.52 g/l)
1985	*	**14.7**	3.04	1.54	2	1.21
1926	*	**13.8**	**4.78**	**0.44**	**58**	**1.93**
1943	*	12.9	**6.35**	1.40	**17**	**1.88**
1928	*	11.9	**4.15**	2.74	**102**	**1.68**
1988	*	**21.3**	1.66	0.75	**58**	0.349
1941	**	**15.2**	**4.94**	**0.43**	**84**	**1.55**
1956	**	**32.6**	2.15	0.70	**195**	0.50
1945	**	**26.8**	2.49	2.55	7	**1.69**
1974	***	**15.8**	2.84	1.22	6	**1.97**
1936	***	**15.9**	**6.06**	1.53	9	1.28
1930	***	**20.0**	**3.54**	1.78	2	**1.82**

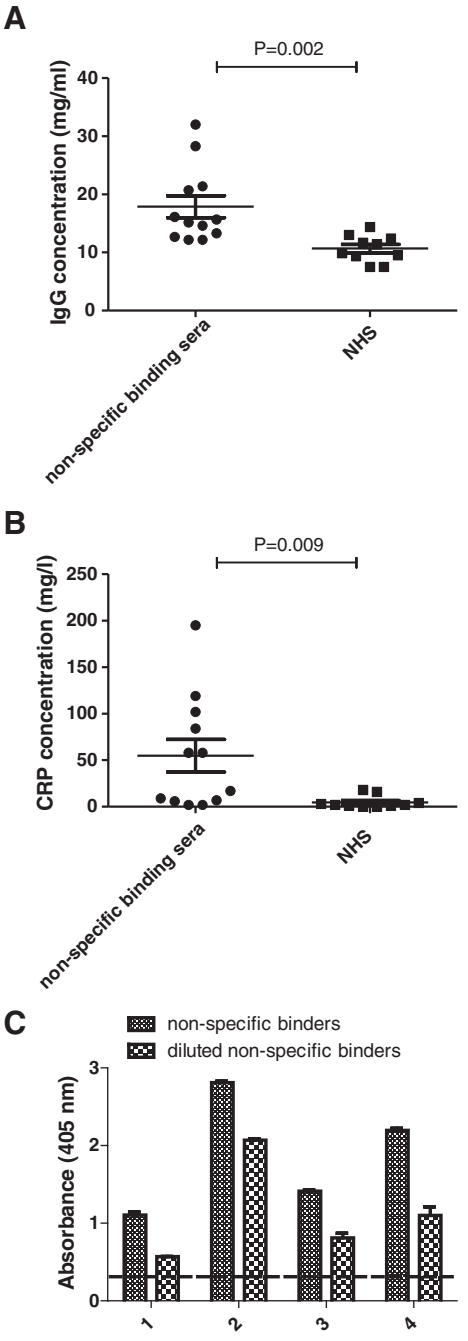

Fig. 5. Inflammatory markers in sera with non-specific binding. The concentration of IgG (A) and C-reactive protein (CRP) (B) was analysed in 11 sera with non-specific binding and in 10 sera without non-specific binding using rate nephelometry. P-values were calculated using a one-tailed student's *t*-test. C) Four sera with elevated concentrations of IgG and a high level of non-specific binding were diluted to the IgG concentration of normal human serum (NHS, 10 mg/ml) and the level of non-specific binding was compared to NHS (stippled line) and undiluted non-specific binding sera.

binding) and heated to slightly elevated temperatures (40 °C, overnight). This revealed that non-specific binding increased with increasing concentration of IgG (Fig. 8).

Table 3
Non-specific binding in sera from patients with immunoglobulins directed against the indicated infectious bacteria. Non-specific binding was defined as IgG deposition on a non-coated, detergent-blocked ELISA well with AUFS (A405/650) above 0.2.

Bacteria	Number of samples	Non-specific binding sera (%)
Yersinia enterocolitica	10	30
Campylobacter jejuni	10	10
Salmonella enteritidis/typhimurium	10	50
Legionella pneumophila	10	40
Helicobacter pylori	10	20
Treponema pallidum[a]	10	70
Bordetella pertussis	10	80

[a] Serum samples were heated to 56 °C for 30 min.

4. Discussion

4.1. Non-specific binding and false positive ELISA results

False positive ELISA results are a major problem in the detection of human specific immunoglobulins. We assessed the extent of the problem by analysing 847 sera for non-specific immunoglobulin deposition on a non-coated surface. Overall, 4–32% of the sera showed relative non-specific binding, depending on the assay. Non-specific binding is thus a significant problem and should be addressed in all routine laboratories measuring human (auto)antibodies.

The non-specific immunoglobulin deposition was found to be dependent on the assay conditions and we evaluated the individual steps of the ELISA setup in order to minimize the level of non-specific binding.

4.2. The effect of blocking agents

We found that insufficient blocking of the ELISA plate could cause non-specific binding and that antibodies directed against the blocking agent (most commonly BSA and skimmed milk) could result in false positive results. Previous studies have indicated a high level of BSA antibodies in sera from some blood donors and patients suffering from systemic autoimmune diseases (Jørgensen et al., 2005). Skimmed milk

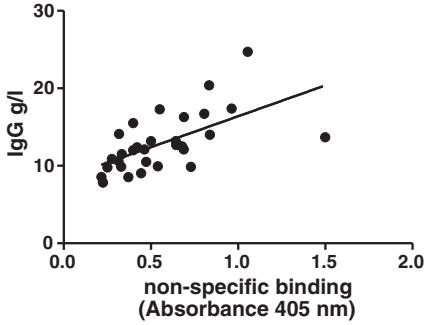

Fig. 6. 30 serum samples positive for antibodies against *Bordetella pertussis* (10 sera), *Treponema palladium* (10 sera) and *Salmonella* sp. (10 sera) were analysed for the level of non-specific binding (x-axis) and IgG concentration (y-axis). The level of non-specific binding and IgG concentration was found to correlate with a slope of 8.0 ± 2.0 (R-square = 0.37, P = 0.004).

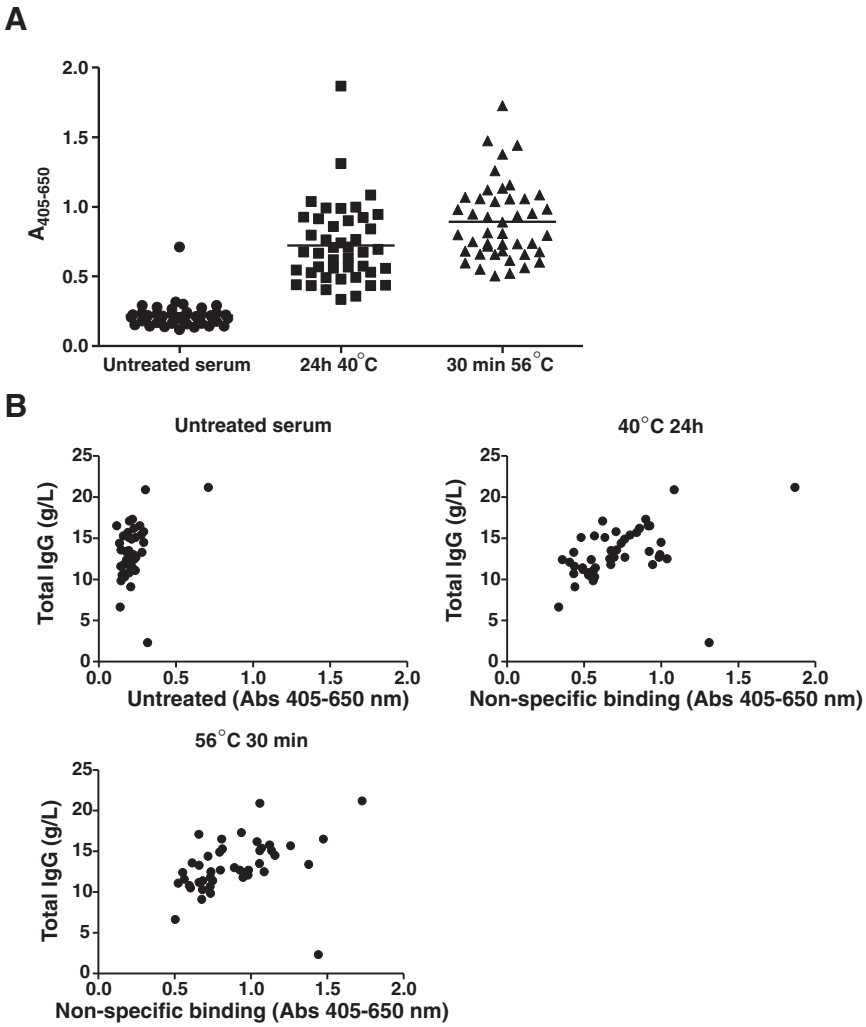

Fig. 7. Induction of nonspecific binding in patient sera. The level of non-specific binding was analysed in 44 serum samples with antibodies to bacteria before and after heating to 40 °C overnight or 56 °C for 30 min (A) and the level of non-specific binding was compared to IgG concentration (B).

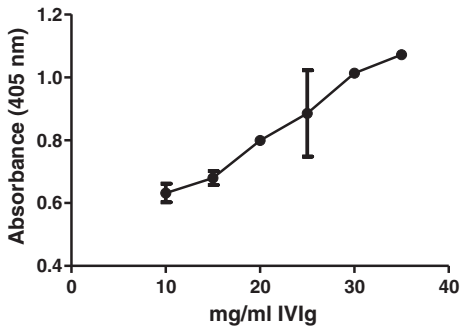

Fig. 8. Non-specific binding can be induced in NHS. Non-specific binding in NHS with elevated IgG concentration is dependent on IgG concentration. IVIg was added to NHS to elevate total IgG concentration to the indicated concentration and incubated at 40 °C for 24 h. Nonspecific IgG deposition was determined using the ELISA setup described in Materials and methods.

is also a commonly used blocking agent and antibodies in sera from individuals with milk antibodies may cause immunoglobulin deposition.

As polysorbate 20 was found to block ELISA wells with equal efficiency as the used protein blocking agents, detergent blocking was preferred over protein blocking. A number of supplementations were also assessed for their ability to inhibit non-specific binding in the incubation buffer. 1% of detergent (polysorbate 20/Triton X-100), animal sera (calf/rabbit), purified protein (ovalbumin/BSA) and skimmed milk were compared as buffer additives. Non-specific IgG binding could be markedly suppressed, especially using 1% skimmed milk or 1% ovalbumin. Ovalbumin however, also induced some non-specific binding in the control sera, possibly as a result of ovalbumin/anti-ovalbumin complex formation. Skimmed milk reduced the non-specific binding approximately 50% without increasing non-specific binding in control sera. Also, sera were diluted and pre-incubated on a

non-coated surface and this was also found to decrease the non-specific binding.

4.3. The effect of the conjugate

The IgG deposition was confirmed using different AP-conjugated secondary antibodies directed against whole IgG, gamma chain or kappa chain showing that non-specific binding was not caused by binding of the secondary antibody (results not shown). IgG deposition was also confirmed using AP-conjugated protein G, ruling out the possibility of interfering anti-animal antibodies (Kricka, 1999).

4.4. The role of immune-complexes and Ig concentration

ICs are known to deposit non-specifically on polystyrene surfaces, and we observed that some but not all sera with non-specific binding (four out of 16 sera) contained C1q-binding ICs. ICs were prepared and added to a serum not showing non-specific IgG deposition. Addition of ICs was found to induce non-specific binding in a concentration-dependent manner. We also measured the concentration of IgG, IgA and IgM in 11 serum samples with non-specific binding showing that all had increased immunoglobulin concentrations. Nine out of 11 had increased IgG concentration; six out of 11 had increased IgA levels, whereas all samples had IgM concentrations in the normal range or below.

4.5. Non-specific binding and inflammatory parameters

CRP concentrations were found to be markedly increased together with immunoglobulins. Elevated IgG concentrations are seen in systemic autoimmune diseases, but elevated IgG levels are also observed in infections, lymphoproliferative disorders and non-haematological malignancies (Dispenzieri et al., 2001). 70 serum samples from patients with recent or ongoing bacterial infections (antibody positive) were analysed, showing that 43% showed non-specific binding. We analysed additional 30 sera with suspected infectious background for non-specific binding and immunoglobulin concentration showing a correlation between IgG concentration and non-specific binding. Immunoglobulin concentration in itself was, however, not found to be the only cause of non-specific binding as non-specific binding sera with elevated IgG still showed non-specific binding after dilution to the level of NHS (10 mg/ml). Some of the serum samples positive for bacterial antibodies had been heat-treated to 56 °C and 44 new samples without prior heat treatment was analysed for non-specific deposition of IgG. 43 of 44 samples showed initially little non-specific binding but the non-specific could be heat-induced and the level of non-specific binding was dependent on the IgG concentration. This suggests that elevated post sampling temperatures may cause massive problems for nonspecific binding. Also, IgG was added to NHS without non-specific binding showing that increased immunoglobulin concentration alone was not sufficient to induce non-specific binding.

4.6. The effect of temperature

Since non-specific binding sera were observed to contain elevated levels of immunoglobulins and since non-specific binding could be induced by heat-aggregated IVIg, we investigated if the IgG modification needed to induce non-specific binding could occur under physiological conditions or conditions occurring in sera preanalytically but after sampling. IVIg was added to NHS and the immunoglobulin-spiked serum was treated with temperatures found in the body under fever or experienced during transport or storage of sera (40 °C), where it was observed to induce non-specific binding dependent on IgG concentration. This experiment thereby provided an explanation for the non-specific binding observed, suggesting that conformational alterations occur in immunoglobulins at high concentrations and under elevated temperatures (either elevated body temperature or elevated temperature for the blood sample pre-analytically).

4.7. Conclusion

ELISA can quantify minute amounts of antibodies present in serum. However, ELISA holds some problems with regard to specificity and false positive results may occur, causing autoantigen-negative sera with non-specific binding to be interpreted as antigen-positive (Cole et al., 1999; Despres and Grant, 1998). Non-specific binding can be quantified using non-coated, blocked wells and the signal can be subtracted from the antigen-specific signal before the presence of autoantibodies is reported. Also, alternative methods (e.g. indirect immunofluorescence or immunoblots) can in many cases be used to confirm ELISA results. Elevated levels of immunoglobulins have previously been observed in sera showing false positive test results in ELISA systems (McFarlane et al., 1990; Cowchock et al., 1988).

In conclusion, we found increased levels of immunoglobulins (IgG, IgA) in samples showing non-specific binding and we propose that non-specific binding occurs in serum samples with elevated immunoglobulin concentration exposed to elevated temperatures. We suggest that all sera tested for antigen-specific antibodies in solid phase immunoassays should also be analysed for non-specific binding immunoglobulins, which may be formed in vivo during fever or in vitro upon storage at too high temperature.

Acknowledgements

Inga Laursen passed away 11.12.2011. We thank her family for permission to publish her work.

References

Andersen, D.C., Koch, C., Jensen, C.H., Skjødt, K., Brandt, J., Teisner, B., 2004. High prevalence of human anti-bovine IgG antibodies as the major cause of false positive reactions in two-site immunoassays based on monoclonal antibodies. J. Immunoass. Immunochem. 25, 17.

Andersson, J., Ekdahl, K.N., Larsson, R., Nilsson, U.R., Nilsson, B., 2002. C3 adsorbed to a polymer surface can form an initiating alternative pathway convertase. J. Immunol. 168, 5786.

Butler, J.E., 2000. Enzyme-linked immunosorbent assay. J. Immunoassay 21, 165.

Cohen Tervaert, J.W., Damoiseaux, J., 2012. Antineutrophil cytoplasmic autoantibodies: how are they detected and what is their use for diagnosis, classification and follow-up? Clin. Rev. Allergy Immunol. 43, 211.
Cole, L.A., Rinne, K.M., Shahabi, S., Omrani, A., 1999. False-positive hCG assay results leading to unnecessary surgery and chemotherapy and needless occurrences of diabetes and coma. Clin. Chem. 45, 313.
Cowchock, S., Fort, J., Munoz, S., Norberg, R., Maddrey, W., 1988. False positive ELISA tests for anticardiolipin antibodies in sera from patients with repeated abortion, rheumatologic disorders and primary biliary cirrhosis: correlation with elevated polyclonal IgM and implications for patients with repeated abortion. Clin. Exp. Immunol. 73, 289.
Despres, N., Grant, A.M., 1998. Antibody interference in thyroid assays: a potential for clinical misinformation. Clin. Chem. 44, 440.
Dispenzieri, A., Gertz, M.A., Therneau, T.M., Kyle, R.A., 2001. Retrospective cohort study of 148 patients with polyclonal gammopathy. Mayo Clin. Proc. 76, 476.
Engvall, E., Perlmann, P., 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8, 871.
Hernandez-Molina, G., Leal-Alegre, G., Michel-Peregrina, M., 2011. The meaning of anti-Ro and anti-La antibodies in primary Sjogren's syndrome. Autoimmun. Rev. 10, 123.
Hu, B., Even, C., Plagemann, P.G., 1992. Immune complexes that bind to ELISA plates not coated with antigen in mice infected with lactate dehydrogenase-elevating virus: relationship to IgG2a- and IgG2b-specific polyclonal activation of B cells. Viral Immunol. 5, 27.
Jethwa, H.S., Nachman, P.H., Falk, R.J., Jennette, J.C., 2000. False-positive myeloperoxidase binding activity due to DNA/anti-DNA antibody complexes: a source for analytical error in serologic evaluation of anti-neutrophil cytoplasmic autoantibodies. Clin. Exp. Immunol. 121, 544.
Jørgensen, C.S., Hansen, K.B., Jacobsen, S., Halberg, P., Ullman, S., Hansen, D., Mikkelsen, T.L., Weile, B., Madsen, M.H., Wiik, A., Houen, G., 2005. Absence of high-affinity calreticulin autoantibodies in patients with systemic rheumatic diseases and coeliac disease. Scand. J. Clin. Lab. Invest. 65, 403.
Kaplan, I.V., Levinson, S.S., 1999. When is a heterophile antibody not a heterophile antibody? When it is an antibody against a specific immunogen. Clin. Chem. 45, 616.
Kricka, L.J., 1999. Human anti-animal antibody interferences in immunological assays. Clin. Chem. 45, 942.
Levinson, S.S., 1992. Antibody multispecificity in immunoassay interference. Clin. Biochem. 25, 77.
Levinson, S.S., Miller, J.J., 2002. Towards a better understanding of heterophile (and the like) antibody interference with modern immunoassays. Clin. Chim. Acta 325, 1.
Lewis, J.G., Florkowski, C.M., Elder, P.A., Hunt, P.J., 2003. Rheumatoid factor and false positive sex-hormone binding globulin. Clin. Chim. Acta 332, 139.
McFarlane, I.G., Smith, H.M., Johnson, P.J., Bray, G.P., Vergani, D., Williams, R., 1990. Hepatitis C virus antibodies in chronic active hepatitis: pathogenetic factor or false-positive result? Lancet 335, 754.
Meroni, P.L., Borghi, M.O., Raschi, E., Tedesco, F., 2011. Pathogenesis of antiphospholipid syndrome: understanding the antibodies. Nat. Rev. Rheumatol. 7, 330.
Moutsopoulos, H.M., Zerva, L.V., 1990. Anti-Ro (SSA)/La (SSB) antibodies and Sjogren's syndrome. Clin. Rheumatol. 9 (Suppl. 1), 123.
Nilsson, U.R., Storm, K.E., Elwing, H., Nilsson, B., 1993. Conformational epitopes of C3 reflecting its mode of binding to an artificial polymer surface. Mol. Immunol. 30, 211.
Peterfi, Z., Kocsis, B., 2000. Comparison of blocking agents for and ELISA for LPS. J. Immunoassay 21, 341.
Reen, D.J., 1994. Enzyme-linked immunosorbent assay (ELISA). Methods Mol. Biol. 32, 461.
Schultz, D.R., Tozman, E.C., 1995. Antineutrophil cytoplasmic antibodies: major autoantigens, pathophysiology, and disease associations. Semin. Arthritis Rheum. 25, 143.
Shovman, O., Gilburd, B., Barzilai, O., Langevitz, P., Shoenfeld, Y., 2007. Novel insights into associations of antibodies against cardiolipin and beta2-glycoprotein I with clinical features of antiphospholipid syndrome. Clin. Rev. Allergy Immunol. 32, 145.
van Weemen, B.K., Schuurs, A.H., 1971. Immunoassay using antigen–enzyme conjugates. FEBS Lett. 15, 232.
Voller, A., Bidwell, D.E., Bartlett, A., 1976. Enzyme immunoassays in diagnostic medicine. Theory and practice. Bull. World Health Organ. 53, 55.
Willman, J.H., Martins, J.H., Jaskowski, T.D., Hill, H.R., Litwin, C.M., 1999. Heterophile antibodies to bovine and caprine proteins causing false-positive human immunodeficiency virus type 1 and other enzyme-linked immunosorbent assay results. Clin. Diagn. Lab. Immunol. 6, 615.
Xiao, Y., Isaacs, S.N., 2012. Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA) — not all BSAs are alike. J. Immunol. Methods 384, 148.