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Research paper

Preventing intense false positive and negative reactions attributed to the principle of ELISA to re-investigate antibody studies in autoimmune diseases

Kuniaki Terato [a,*], Christopher T. Do [a], Dawn Cutler [a], Takaki Waritani [a], Hiroshi Shionoya [b]

[a] Chondrex Inc., 2607 151st Place NE, Redmond, WA 98052, United States
[b] Asama Chemical Co. Ltd, Chuo-ku, Tokyo, Japan

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ABSTRACT

To study the possible involvement of potential environmental pathogens in the pathogenesis of autoimmune diseases, it is essential to investigate antibody responses to a variety of environmental agents and autologous components. However, none of the conventional ELISA buffers can prevent the false positive and negative reactions attributed to its principal, which utilizes the high binding affinity of proteins to plastic surfaces. The aims of this study are to reveal all types of non-specific reactions associated with conventional buffer systems, and to re-investigate antibody responses to potential environmental pathogenic and autologous antigens in patients with autoimmune diseases using a newly developed buffer system "ChonBlock™" by ELISA.

Compared to conventional buffers, the new buffer was highly effective in reducing the most intense false positive reaction caused by hydrophobic binding of immunoglobulin in sample specimens to plastic surfaces, "background (BG) noise reaction", and other non-specific reactions without interfering with antigen–antibody reactions. Applying this buffer, we found that IgG antibody responses to *Escherichia coli* O111:B4, *E. coli* lipopolysaccharide (LPS) and peptidoglycan polysaccharide (PG-PS) were significantly lower or tended to be lower in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), whereas IgA antibody responses to these antigens were equal or tended to be higher compared to normal controls. As a consequence, the IgA/IgG antibody ratios against these agents were significantly higher in patients with RA and SLE, except for Crohn's disease, which showed significantly higher IgG responses to these antigens.

To assay antibodies in human sera, it is indispensable to eliminate false positive and negative reactions by using an appropriate buffer system, and to include antigen non-coated blank wells to determine BG noise reactions of invidual samples. Finally, based on our preliminary analysis in this study, we propose that low IgG antibody responses to potential pathogenic environmental factors may be the fundamental disorder in autoimmune diseases.

1. Introduction

A variety of immunoassay systems, such as ELISA, are widely used for assaying antibodies and antigens at high sensitivity using micro-titer plates or small bead particles as a carrier of target molecules. Unfortunately, this system has been used without fully comprehending the numerous vexing

* Corresponding author.
E-mail addresses: terato@chondrex.com (K. Terato), cdo@chondrex.com (C.T. Do), dcutler@chondrex.com (D. Cutler), twaritani@chondrex.com (T. Waritani), h.shionoya@asama-chemical.co.jp (H. Shionoya).

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phenomena attributed to the principle, which utilizes high binding affinity of proteins to plastic surfaces as we reported previously (Fujii et al., 1989).

Indeed, none of the conventional ELISA buffer systems currently used can be applied for assaying antibodies in human sera due to the poor blocking effect on non-specific reactions, especially the most intense false positive reaction caused by hydrophobic binding of immunoglobulin components in sample specimens to plastic surfaces or 'BG noise reaction'. Although the unique BG noise reaction of individual samples can be determined by including control wells not coated with antigen, these control wells were and still are frequently omitted in plates made by investigators conducting basic research, and are not even included in commercially prepared plates meant for automated analysis. Consequently, data influenced largely by a variety of false positive reactions have led to numerous uncertain conclusions and misunderstandings. In some instances, OD values of control wells can be as high as the values in antigen-coated wells regardless of the antigen (Fujii et al., 1989; de Vries et al., 1992), and more importantly, BG noise values in autoimmune disease groups are higher than those in normal controls (Fujii et al., 1989).

To solve this fundamental problem in ELISA, we previously developed an improved antibody assay system using a buffered heterologous serum (rabbit and goat) as a blocking and sample dilution buffer (Fujii et al., 1989) and applied it to analyze anti-collagen antibodies in RA (Terato et al., 1990, 1996). Unfortunately, this protocol cannot be applied to assay antibodies against environmental agents, since heterologous serum contain antibodies, which share similar antigen specificity and competitively inhibit relevant antibodies in test samples.

Therefore, it is absolutely imperative to have an appropriate assay system, which eliminates these false positive and negative reactions involved in ELISA for any field of study. In this study, we initially re-investigated the individual types of non-specific reactions in conventional ELISA systems, and then evaluated the blocking efficacy of a newly developed ELISA buffer, ChonBlock™ (Chondrex Inc., Redmond, WA), from every possible angle.

Lastly, we applied the new buffer system to assay antibody levels against a variety of environmental agents in human sera to study the possible involvement of potential environmental pathogens in the pathogenesis of autoimmune diseases. In this preliminary study, we found that IgG antibody responses to potential pathogenic antigens, such as *Escherichia coli*, lipopolysaccharide (LPS) from *E. coli*, and also peptidoglycan polysaccharide (PG-PS) from *Streptococcus pyogenes*, are significantly lower or tended to be lower. As a consequence, the IgA/IgG antibody ratios against these antigens are significantly higher in patients with RA and SLE compared to normal controls. In contrast, significantly high IgG antibody responses to these pathogens and LPS produced by *Porphyromonas gingivalis* (Pg-LPS) were observed in patients with Crohn's disease.

2. Materials & methods

2.1. Human serum samples

The human serum samples used for this study were from 15 normal controls, 61 patients with RA, 7 patients with SLE and 7 patients with Crohn's disease. Among these, sera from 10 healthy controls, 7 patients with SLE and Crohn's disease were obtained from Bioreclamation LLC, NY, whereas the remaining sera were selected from our frozen stock. An institutional Review Board (IRB) exemption was granted from the Western Institutional Review Board (WIRB), Olympia, WA, USA. According to the WIRB Regulatory Affairs Department, this research project met the conditions for exemption under 45 CFR 46.101(b)(4).

2.2. Microtiter plates

Two types of ELISA plates were used for these studies: a high affinity Immulon 2 HB plate (Thermo Fisher, Waltham, MA) for coating protein antigens and *E. coli* by hydrophobic binding, and Costar DNA binding plate (Corning Inc, Corning, NY) for coupling CCP and a control peptide by covalent binding.

2.3. ELISA buffers and reagents

Seven different buffers were used to compare their blocking effects on the hydrophobic binding of human serum immunoglobulin components to plastic surfaces: 1) 0.1 M Tris-Saline Buffer, pH 7.8, containing 0.05% Tween 20 (TSB-Tween), 2) 1% BSA in TSB-Tween (BSA-Tween), 3) 2% milk casein enzyme hydrolysate in TSB-Tween (Milk-Tween), 4) radioimmunoassay buffer (RIA) containing 1% BSA, 350 mM NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate and 0.1% sodium dodecyl sulfate (SDS) in 0.01 M Tris–HCl buffer, pH 7.6, 5) RIA supplemented with 10% normal goat serum (RIA-10% NGS), 6) undiluted buffered normal goat serum with TSB (100% NGS) and 7) ChonBlock™, a protein-based blocking/sample dilution buffer, not containing any biologically or immunologically active components. In addition, Triton X buffer provided in a commercially available CCP antibody assay kit (BioRad, Hercules, CA) was used for an inhibition test of anti-CCP antibodies.

HRP-conjugated goat anti-human IgG (Fc-specific: Cat # 2047-5) (Southern Biotech, Birmingham, AL) and anti-human IgA antibodies (alpha-specific: Cat # 14-10-01) (KPL, Gaithersburg, MD) were used for detecting human serum IgG and IgA, respectively. These detection antibodies were diluted at 1/12,000 and 1/1500, respectively, by ChonBlock™ detection antibody dilution buffer otherwise indicated.

2.4. Synthesis of peptide antigens

Two cyclic peptides, a cyclic citrullinated peptide (CCP)(cfc1-cyc: HQCHQESTXGRSRGRCGRSGS, circled between two Cs, X: Citrulline) corresponding with filaggrin (306–324), a filament associated protein, and a negative control cyclic peptide (cf0-cyc: HQCHQESTRGRSRGRCGRSGS, circled between two Cs), were synthesized at Biosynthesis (Lewisville, Texas) according to the method described by Schellekens et al. (Schellekens et al., 2000) and Perez et al. (Pérez et al., 2006).

2.5. Coating microtiter plates with antigen

Protein antigens were dissolved in PBS, pH 7.4, at 5 μg/ml, and added to Immulon 2 HB plate at 100 μl/well. The plates were incubated overnight at 4 °C. An *E. coli* (heat killed) suspension (OD600: 10) supplied by Asama Chemicals (Tokyo, Japan) was diluted 1/100 with distilled water, and added to

Immulon 2HB plate at 50 μl/well. The plates dried overnight at room temperature.

Synthetic peptides and IgG free BSA (Jackson Immuno-Research, West Grove, PA) were dissolved at 10 μg/ml in 0.05 M $NaHCO_3$, and added to Costar DNA Binding Plates at 100 μl/well. The plates were incubated overnight at 4 °C to allow covalent binding of the peptide to the plastic surfaces. To measure anti-CCP antibodies using the RIA-10% NGS buffer system, the control wells were coupled with glycine instead of IgG-free BSA used by Schellekens et al. (Schellekens et al., 2000) and Perez et al. (Pérez et al., 2006), because human sera contain antibodies that bind strongly with BSA (Andersen et al., 2004) as described later. All antigen treated plates were washed six times with cold LPS-free filtered water, dried at room temperature and stored at 4 °C until use.

2.6. Inhibition test

Inhibition of anti-CCP antibodies: Serum from RA #3 was diluted at 1/800 in RIA-10% NGS, Triton X buffer and ChonBlock™, respectively, and 0.8 ml of this solution was mixed with 0.2 ml of CCP- and control-peptide (0.1–1.0 mg/ml in distilled water) solutions, respectively. After overnight incubation at 4 °C, the samples were assayed in non-antigen and CCP-coupled wells.

Inhibition of anti-*E. coli* antibodies: An *E. coli* suspension in distilled water (OD650 value: 10) was dispended into micro-centrifuge tubes by 100, 50, 25, 12.5, 6.25 and 3.125 μl, and spun at 10,000 rpm for 5 min. A human serum sample (NL #2) was diluted at 1/100 with 1% BSA-Tween, 100% NGS and ChonBlock™, respectively, and 100 μl of individual solution was added to *E. coli* pellets, and incubated overnight at 4 °C. The supernatant was further diluted to 1/2500 with corresponding buffer, and assayed in antigen non-coated and *E. coli*-coated Immulon 2HB plates.

2.7. Standards for human serum antibody assays

Standards for assaying antibodies against individual antigens were prepared from selected human sera. The highest dose of standard was adjusted to give 3.0 ± 0.1 of OD 450 value, and defined as 32 units/ml. For example, for IgG antibody assays, a normal serum, NL # 2, was diluted as follows depending on antigens, *E. coli*: 1/2500, LPS: 1/300, PG-PS: 1/5000, Pg-LPS: 1/800, ovalbumin (OVA): 1/2000, BSA: 1/1500 and bovine IgG: 1/4000. In addition, for IgG anti-CCP antibody assay, a combined serum from 25 anti-CCP-antibody positive patients with RA was diluted at 1/500. For IgA antibody assays, sera from 3 normal individuals absorbed by protein G as described later were diluted depending on antigens, LPS: NL #5 diluted at 1/250, *E. coli*: NL #8 diluted at 1/2000, and PG-PS: NL #8 diluted 1/4000. Since serum IgA anti-Pg-LPS antibody levels were generally low, NL #8 diluted at 1/500 was used as 8 units/ml.

2.8. ELISA procedure for human serum antibody assay

For IgG antibody assays, human serum samples were diluted with ChonBlock™ sample dilution buffer depending on antigens: *E. coli* (1/10,000), LPS (1/1000), PG-PS (1/20,000), Pg-LPS (1/1000), OVA (1/5000), BSA (1/500), bovine IgG (1/20,000), and CCP (1/500). For IgA antibody assays, all serum specimens including standards were absorbed with Protein G to remove IgG as follows. Serum was diluted at 1/100 with ChonBlock™ sample dilution buffer, and 100 μl of the diluted serum was then mixed with 100 μl of Protein G suspension (30%) in PBS, incubated 10 min at room temperature, and spun at 10,000 rpm for 3 min with a table top centrifuge. The supernatants were further diluted with ChonBlock™ sample dilution buffer depending on antigens: *E. coli* (1/4000), LPS (1/1000), PG-PS (1/10,000) and Pg-LPS (1/1000).

Plates were blocked at room temperature for 1 h with ChonBlock™ sample dilution buffer just before use, and washed with PBS-Tween. Then, 100 μl of diluted test samples was added into antigen non-coated control wells and antigen-coated wells, and incubated at room temperature for 2 h. After washing the plate with PBS-Tween, 100 μl of HRP-conjugated detection antibody diluted by ChonBlock™ detection antibody dilution buffer was added into all wells, and reacted at room temperature for 2 h. Color was developed at room temperature for 25 min by adding 100 μl of TMB solution (Cat # 34028, Thermo Scientific, Rockford, Illinois), and stopped by 50 μl of 2N sulfuric acid. The absorbance values were measured at 450/630 nm. The samples, which gave higher OD values than the highest standard (32 units/ml), were re-assayed at higher dilution. The results were shown as kilo-units/ml (units/ml).

2.9. Statistic analysis

Data is expressed as the mean ± standard deviation. For statistical evaluation, the unpaired Student's *t*-test was used to determine the significance of differences between normal control and disease groups. Results were shown using *p*-values with 5% level of significance if not otherwise stated.

3. Results

3.1. Intense false positive reaction caused by hydrophobic binding of human serum immunoglobulin components to plastic surfaces

In an ELISA, four types of false positive reactions can be encountered regardless of the antigens coated on the ELISA plate: 1) non-specific reaction caused by the secondary antibody, 2) hydrophobic binding of immunoglobulin components in sample specimens to plastic surfaces, 3) ionic interaction between immunoglobulin in sample specimens and antigen, and 4) immune-recognition of blocking agents by antibodies in serum specimens (e.g. human anti-BSA and anti-bovine IgG antibodies). In addition, two false negative reactions are considered: 1) the competitive inhibition of test antibodies by relevant antibodies present in animal serum, such as NGS, which is added to the sample dilution buffer, and 2) denaturation of enzymes conjugated to detection antibodies, antigens and antibodies by SDS contained in RIA and RIA-10% NGS buffer.

Among these, the hydrophobic binding of immunoglobulin in sample specimens to plastic surfaces causes the most intense false positive reaction in an ELISA. Therefore, we first compared the blocking efficacy of commonly used buffers and ChonBlock™ against this non-specific reaction. For this study, serum from a patient with RA (RA#13) was serially diluted with seven different buffers, and reacted with

non-treated blank wells. As shown in Fig. 1a, none of the commonly used buffers (TSB, RIA, RIA-10% NGS, 1% BSA-Tween, and 2% milk casein) was effective in reducing the binding of human immunoglobulin to the Immulon 2 HB plate. Compared to these buffers, 100% NGS and ChonBlock™ effectively prevented the binding of human immunoglobulin to plastic surfaces, and notably ChonBlock™ was 2–3 time more effective than 100% NGS (Fig. 1a). Similar results were observed in a Costar Covalent plate (Fig. 1b). Interestingly, RIA-10% NGS was more effective in the Costar plate than the Immulon 2 HD plate.

To confirm these results, 10 sera from normal controls and 10 sera form patients with RA were diluted at 1/200 with different buffers, and added to unblocked antigen non-coated plain wells of Immulon 2 HB plates to accentuate the non-specific reactions. As shown in Fig. 2a, both normal and RA sera diluted with 1% BSA-Tween and RIA-10% NGS gave significantly high BG noise values compared to samples diluted by 100% NGS and ChonBlock™. Importantly, the BG noise values of RA sera were significantly higher than those of normal controls ($p < 0.05$) in 1% BSA-Tween as reported (Fujii et al., 1989). On the other hand, the BG noise values of RA sera diluted with RIA-10% NGS were slightly lower than those of normal sera, indicating that the physico-chemical properties of immunoglobulin present in RA sera differ from those of normal controls, and were modestly retarded by the detergents contained in RIA-buffer, such as Triton X, sodium deoxycholate and SDS. Comparable results were observed in the Costar covalent plate (data not shown).

Furthermore, to determine BG noise values in actual assays for IgG and IgA antibodies in human sera, the wells were blocked with ChonBlock™ at room temperature for 1 h, and reacted with the RA sera diluted at 1/100 and 1/1000 with ChonBlock™. As shown in Fig. 2b, BG noise values caused by IgG and IgA in serum specimens were almost completely eliminated when the samples were diluted at 1/100 for IgG, and 1/1000 for IgA antibody assay, although apparent non-specific reactions caused by serum IgA remained at a 1/100 dilution.

3.2. Other false positive and negative reactions encountered with commonly used buffer systems

In addition to the intense false positive reaction caused by the sample itself (BG noise reaction), four other types of non-specific reactions are generally seen in an ELISA. To reveal these false positive and negative reactions in individual buffer systems, two types of antigens, CCP and *E. coli*, were selected as examples, because CCP is widely used as an autologous antigen for diagnostic purposes for RA, whereas *E. coli*, which is a particle antigen with a complex surface structure, is considered to be a typical antigen that strongly interacts with serum immunoglobulin in a non-specific manner. In addition, *E. coli* present in the gastrointestinal tract of animals and humans is consistently interacting with the host's immune system, thus the antibody response to *E. coli* might be a valuable marker for the immune function of individuals.

3.2.1. False positive and negative reactions in anti-CCP antibody assay in various buffer systems

Anti-CCP-antibody positive serum from a patient with RA (#353) was serially diluted with RIA-10% NGS, 100% NGS and ChonBlock™ respectively, and reacted with non-antigen-coupled, control peptide (cf0-cyc)- and CCP (cf1-cyc)-coupled wells of Costar covalent plates. According to the method described by Schellekens et al. (Schellekens et al., 2000) and Perez et al. (Pérez et al., 2006), the plates were blocked with IgG-free BSA in the assay using RIA-10% NGS, whereas the other plates were blocked with the corresponding buffers just before use.

In the RIA-10% NGS system, virtually identical dilution curves were observed in antigen non-coupled plain wells

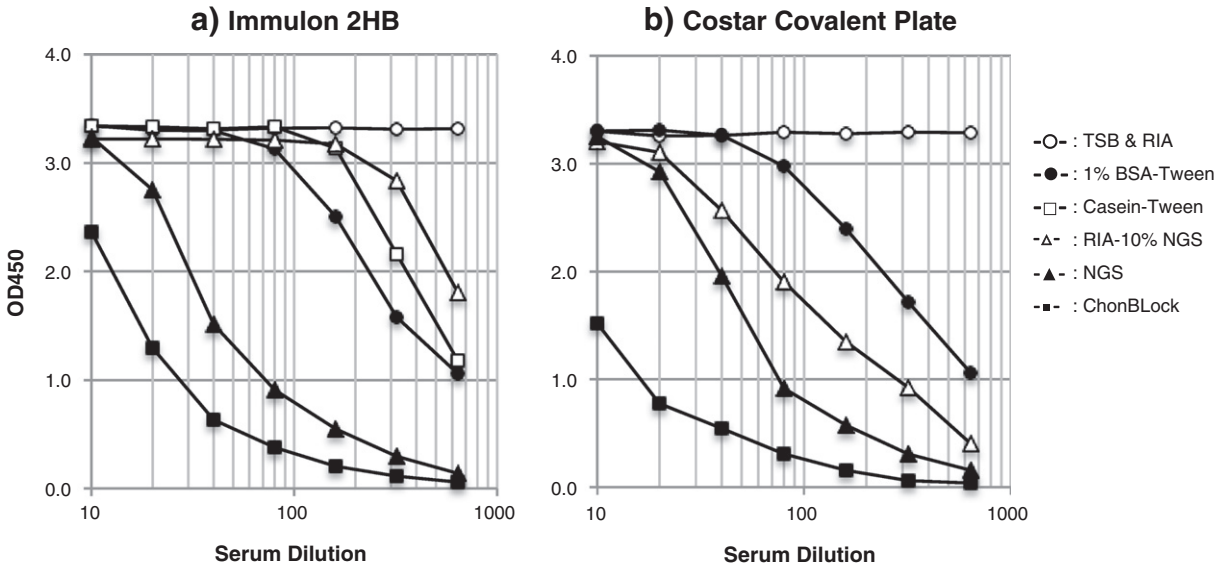

Fig. 1. Hydrophobic binding of human serum immunoglobulin to plastic surfaces: Comparison of blocking effects of various sample dilution buffers. Serum from a patient with RA (RA #3) was serially diluted with individual buffers, and added to antigen non-coated plain wells of high affinity Immulon 2 HD (a) and Costar covalent plates (b). Immunoglobulin bound to the wells was determined by using HRP conjugated goat anti-human IgG antibody.

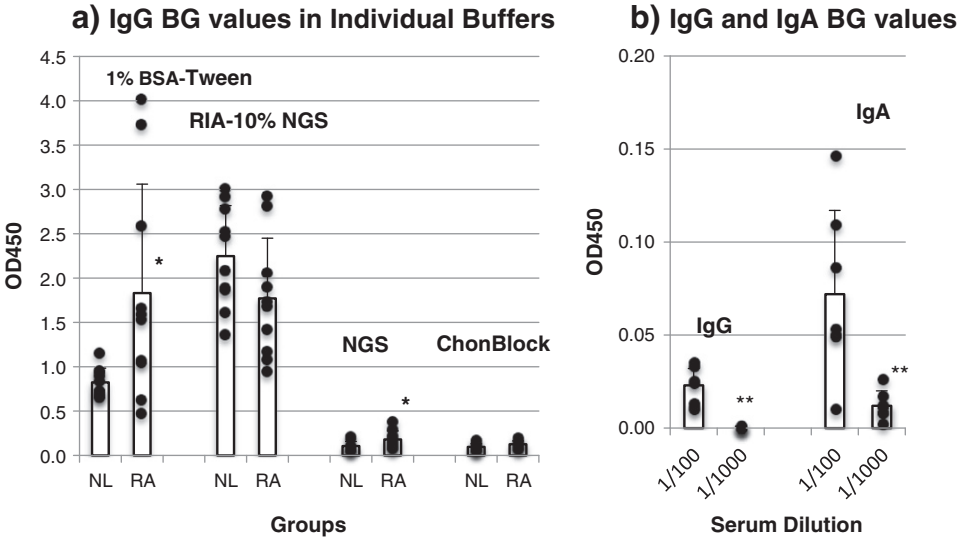

Fig. 2. Comparison of IgG BG noise values of normal and RA sera in different buffer systems (a) and IgG and IgA background noise values of RA sera in the ChonBlock™ buffer system (b). (a) Sera obtained from 10 normal controls (NL) and 10 patients with RA in plain centrifuge tubes were diluted at 1/200 with individual buffers, and added to antigen non-coated plain wells of an Immulon 2 plate, which was not blocked. (b) Sera from 6 patients with RA diluted at 1/100 and 1/1000 with ChonBlock™ were added to plain wells blocked with ChonBlock™, and the IgG and IgA bound to wells were determined with HRP-conjugated goat anti-IgG and IgA antibodies, respectively.

and wells coupled with control peptide and CCP peptide (Fig. 3a–i). Interestingly, OD values in wells lacking antigen, but blocked with BSA (closed square), were higher than the OD values seen in CCP peptide (open circle) and control peptide-coupled wells (closed triangle), indicating that BSA bound to wells was strongly recognized by antibodies present in human serum. Importantly, this result also indicates that BSA bound by SDS in the RIA buffer is not capable of neutralizing the anti-BSA antibodies present in human serum.

Importantly, in the RIA-10% NGS buffer system, the OD values in CCP-coupled wells (Fig. 3a–i) were lower than the corresponding OD values in the ChonBlock™ system (Fig. 3a–iii), indicating that NGS-supplemented to RIA buffer partially inhibited the binding of human serum anti-CCP antibodies to CCP. Indeed, this inhibitory effect of NGS was even more evident when 100% NGS was used (Fig. 3a–ii). In contrast, a good dilution curve with a low background (Fig. 3a–iii) was achieved using the ChonBlock™ system. Furthermore, no reaction was seen when the structurally similar but antigenically unique control peptide was used in the assay using ChonBlock™ (Fig. 3a–iii, closed triangle), confirming the high specificity of anti-CCP antibodies to CCP.

3.2.2. False positive and negative reactions in anti-E. coli antibody assay in various buffer systems

Similarly, significantly divergent dilution curves were observed in anti-*E. coli* antibody assays in the different buffer systems used (Fig. 3b). The OD values in *E. coli* coated wells (open circle) in the 1%BSA-Tween system (Fig. 3b–i) were significantly higher than those seen in other buffer systems (Fig. 3b–ii and iii). As expected, 100% NGS significantly inhibited the detection of anti-*E. coli* antibodies (Fig. 3b–i), whereas ChonBlock™ provided a clear dilution curve (Fig. 3b–iii) with low background.

3.3. Inhibition studies for confirming assay specificity and accuracy of anti-CCP and anti-E. coli antibody assays

To resolve these apparent discrepancies between the assay data obtained by different buffer systems, inhibition tests were performed with the corresponding buffers (Fig. 4). Using the RIA-10% NGS buffer system, an apparent inhibition (approximately 80%) by CCP was observed (data not shown). However, the BG noise values in antigen non-coated control wells were higher than the OD values in antigen-coated wells, rendering the data uninterpretable. Therefore, Triton X buffer, which is provided in a commercial anti-CCP antibody assay kit, was used in subsequent inhibition studies. Interestingly, the Triton X buffer significantly reduced the BG noise value (Fig. 4a, closed triangle), however only a 53% of inhibition by CCP was achieved (Fig. 4a, open triangle). In contrast, nearly complete inhibition (92%) was achieved with the ChonBlock™ buffer system without any non-specific BG noise reaction (Fig. 4a, open circle). As anticipated, the control peptide (Fig. 4a, open square) had no inhibitory effect, indicating that the human anti-CCP antibodies can be assayed without interference by immunoglobulins in test serum with the ChonBlock™ buffer system.

Likewise, nearly complete inhibition (96%) of anti-*E. coli* antibodies was achieved with ChonBlock™ (Fig. 4b, open circle), whereas only 73% inhibition was obtained with 1% BSA-Tween (Fig. 4b, open triangle), indicating that BSA was incapable of preventing the non-specific interaction between immunoglobulins in test samples and *E. coli* particles.

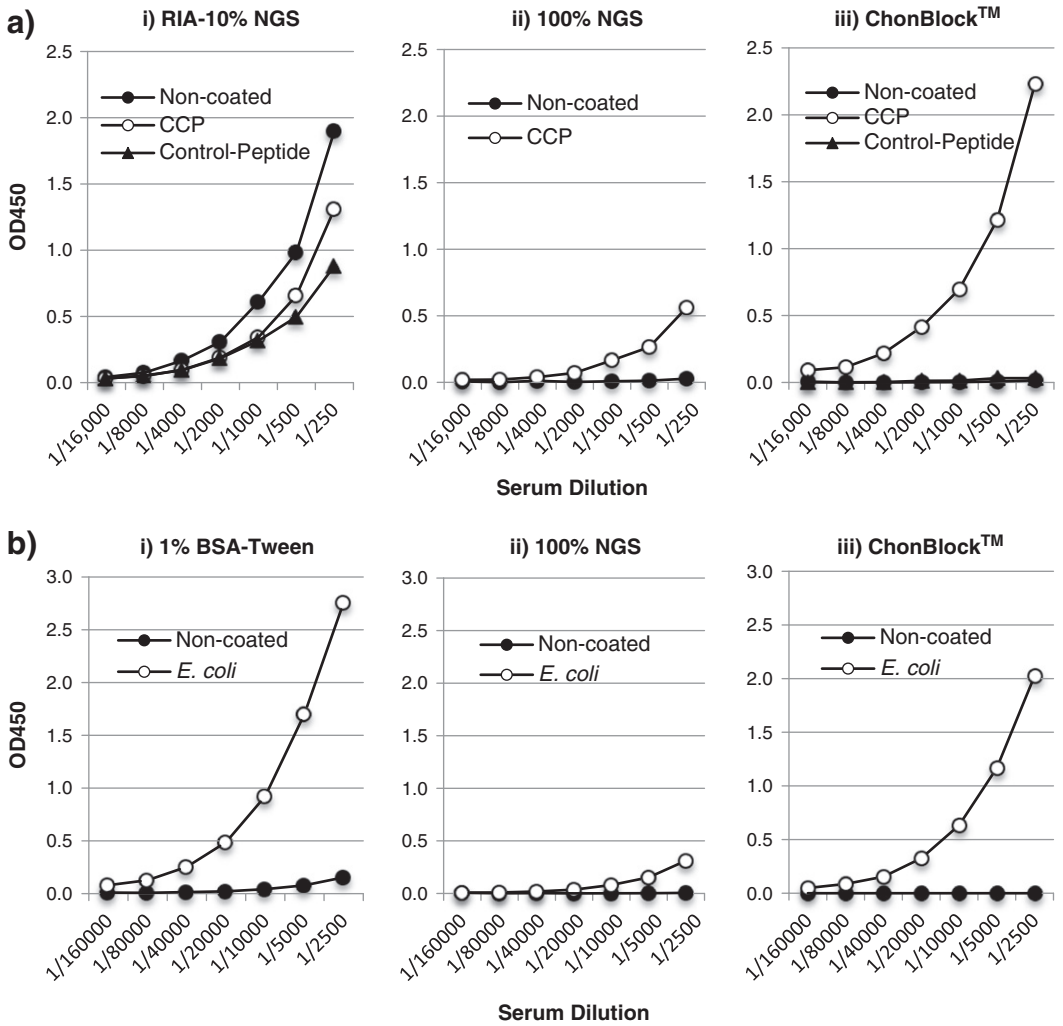

Fig. 3. Comparison of anti-CCP (a) and anti-*E. coli* (b) antibody assay results in different buffer systems. (a) Serum from a patient with RA (RA # 353) positive for anti-CCP IgG antibody was serially diluted with RIA-10% NGS, 100% NGS and ChonBlock™, and added to antigen non-coupled, control peptide- and CCP-coupled wells of Costar plates at room temperature for 2 h. All wells were blocked with 2% BSA, 100% NGS, and ChonBlock™, respectively, before adding samples. (b) Serum from a normal control (NL # 2) was serially diluted with 1% BSA-Tween, 100% NGS and ChonBlock™, and reacted with antigen non-coated and *E. coli*-coated Immulon 2 plates at room temperature for 2 h. All wells were blocked with individual buffers before adding samples. IgG antibodies bound to CCP and *E. coli* were determined with an HRP-conjugated goat anti-IgG antibody.

3.4. Confirming beneficial effects of ChonBlock™

Based on these observations, it was apparent that ChonBlock™ provides substantial promise as a highly effective sample dilution buffer for assaying human serum antibodies against a variety of antigens at low and high serum dilutions. To confirm the beneficial effects of ChonBlock™, CCP was chosen as an example of an autologous antigen, and 13 normal and 13 RA sera were assayed at 1/500 dilution using the RIA-10% NGS and the ChonBlock™ buffer systems. In this assay, glycine-coupled wells rather than BSA-blocked wells were used to determine the BG noise values of individual samples.

As shown in Fig. 5a, in the RIA buffer system, similar OD values were observed in all three wells (glycine-, control peptide- and CCP-coupled) for both normal and RA sera, except 2 RA sera. Importantly, if the OD values obtained in CCP-coupled wells are corrected by subtracting the BG OD values obtained in glycine-coupled control wells, the corrected OD values are as low as 0.040 ± 0.066 for normal sera, and 0.377 ± 0.652 for RA sera. As a consequence, no significant difference was observed between normal and RA in the RIA-10% NGS buffer system (Fig. 5a) due to high BG noise reactions and inhibition of anti-CCP antibodies by NGS.

In contrast, when the samples were diluted with ChonBlock™, OD values of normal sera in two control wells and CCP-coupled wells were equally low (Fig. 5b). Similarly, OD values of RA sera in two control wells were low, but apparently high in CCP-coupled wells. Interestingly, when the OD values in CCP-coupled wells are corrected by subtracting the OD values in Gly-coupled wells, the average $\pm$ SD value of normal and RA sera was 0.050 ± 0.041 and 1.253 ± 1.322 ($p = 0.003$), respectively. Importantly, the corrected OD values in 2/13 normal

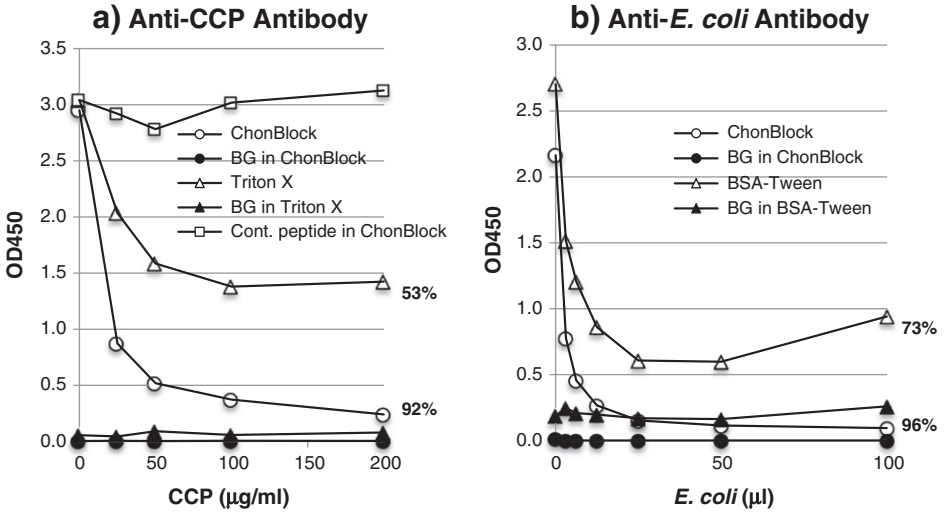

Fig. 4. Inhibition tests of anti-CCP (a) and anti-*E. coli* (b) antibodies in different buffer systems. (a) Serum (RA #3) diluted at 1/800 with RIA-10% NGS, Triton X or ChonBlock™ buffer was mixed with a control-peptide and CCP-peptide solution (0.1–1 mg/ml), respectively as described in the Materials & methods section. After incubation at 4 °C overnight, sample solutions were added to antigen non-coupled and CCP-coupled wells at room temperature for 2 h. b) An *E. coli* suspension in distilled water (OD600 value: 10) was aliquoted into micro-centrifuge tubes and mixed with human serum (NL #2) diluted at 1/100 with 1% BSA-Tween or ChonBlock™, and incubated at 4 °C overnight as described in the Materials & methods section. The supernatant was then diluted to 1/2500 with individual buffers, and added to antigen non-coated and *E. coli*-coated Immulon 2 plates at room temperature for 2 h. IgG antibodies bound to CCP and *E. coli* were determined with an HRP-conjugated goat anti-IgG antibody.

sera and 11/13 RA sera were higher than this normal range, and considered positive for anti-CCP antibodies (see CCP-Gly of normal in Fig. 5b). These results clearly indicate the importance of determining BG noise values of individual samples, and to correct the OD values in antigen-coated wells by subtracting the OD values in control wells regardless of the antigen and buffer system used, especially at low serum dilutions.

3.5. Measuring IgG and IgA antibody responses against potential pathogenic agents in autoimmune diseases by ELISA using the ChonBlock™ buffer system

To study a possible involvement of environmental agents in the pathogenesis of autoimmune diseases, IgG and IgA antibody levels to potential pathogenic environmental agents (*E. coli*, LPS, PG-PS and Pg-LPS) and IgG antibody responses to

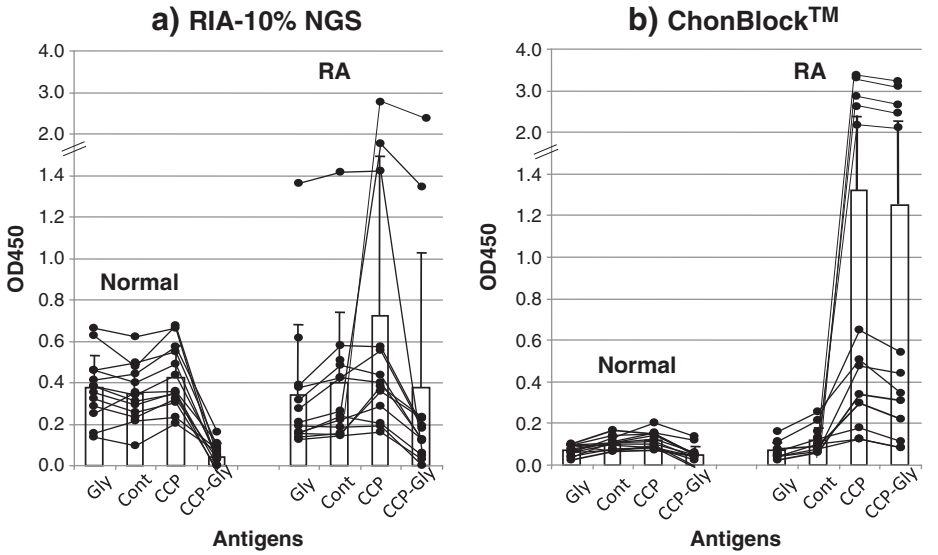

Fig. 5. Comparison of anti-CCP antibody assay results using RIA-10% NGS (a) and ChonBlock™ buffer (b) systems. Sera from 13 normal controls and 13 patients with RA were diluted at 1/500 by a) RIA-10% NGS and b) ChonBlock™ buffer, respectively, and reacted with glycine-, control peptide- and CCP-coupled wells. Glycine was used as control antigen instead of BSA to determine the BG noise values of individual samples. The OD values obtained in CCP-coupled wells were corrected by subtracting the BG noise OD values in glycine-coupled wells (CCP-Gly). All wells were blocked with individual buffers instead of BSA.

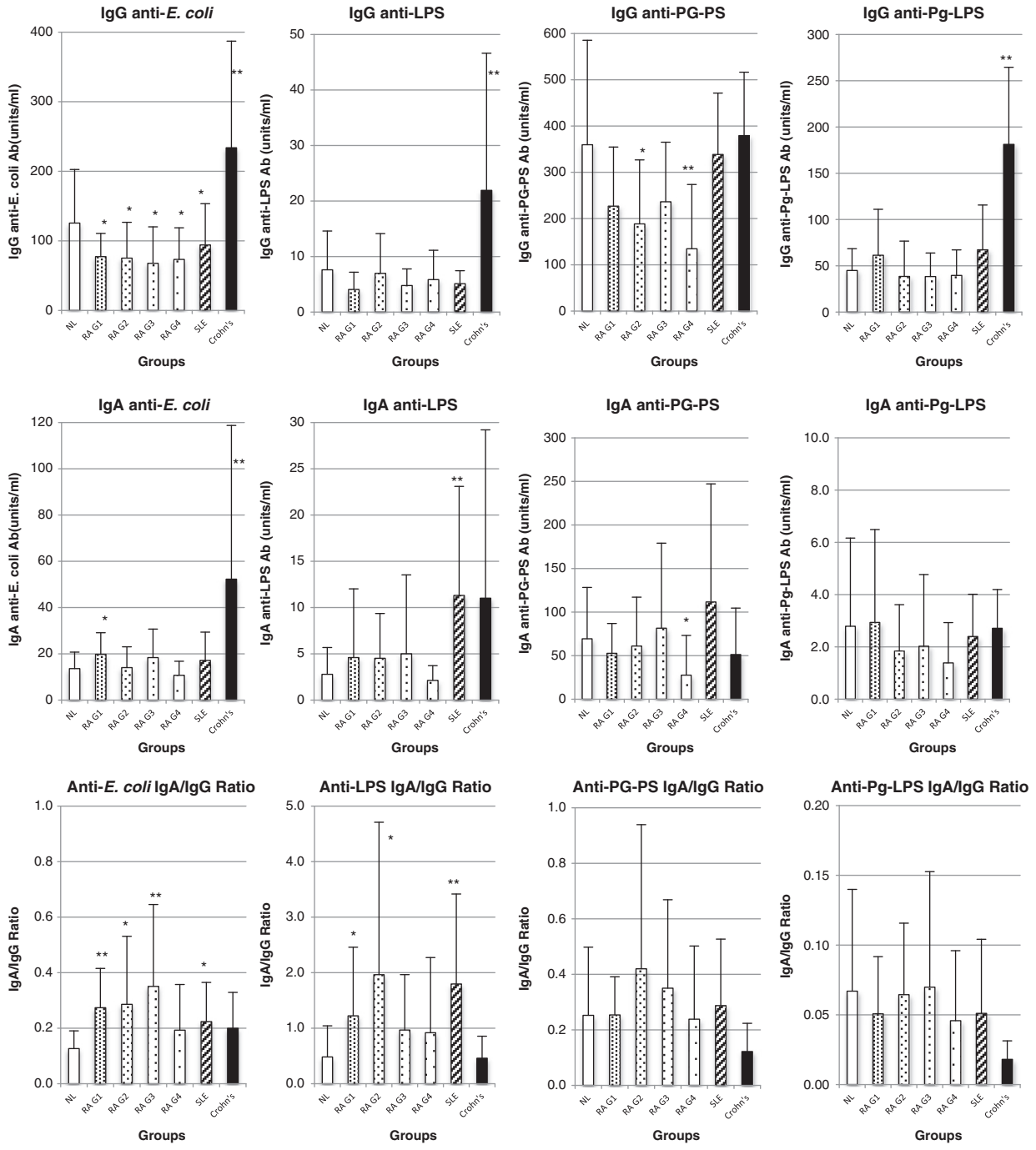

Fig. 6. IgG and IgA antibody levels to potential pathogenic environmental agents in normal controls and patients with RA, SLE, and Crohn's disease. Serum samples were diluted with ChonBlock™ at 1/1000 to 1/20,000 depending on antigens, and assayed for IgG and IgA antibodies, respectively, as described in the Materials & methods section, *: $p > 0.05$, **: $p > 0.01$ (compared to normal controls).

non-pathogenic dietary proteins (OVA, BSA, and bovine IgG) were measured in sera from 16 normal controls, 61 patients with RA, 7 patients with SLE, and 7 patients with Crohn's disease by ELISA using the ChonBlock™ buffer system. Since RA is believed to be polyvalent diseases rather than a single disease influenced by genetic backgrounds, RA patients were divided into 4 groups depending on HLA-haplotypes, G1: 15 patients with homozygous DR4 (O401/0405, 0404, 0408), G2: 16 patients with heterozygous DR1 (01/03, 0707, 0901, 11, 13, 15/16), G3: 16 patients with miscellaneous HLA types, and G4: 14 patients with heterozygous DR4 (0401/01, 03, 0403, 11/12, 15/16).

As shown in Fig. 6a, IgG antibody responses to *E. coli* were significantly lower in all 4 RA groups and SLE compared to

those of normal controls. Similarly, IgG antibody responses to LPS and PG-PS tended to be lower in RA. In contrast, IgG antibody responses to these antigens including Pg-LPS were significantly higher in Crohn's disease. A similar trend was observed in IgG antibody responses to dietary proteins (OVA, BSA, and bovine IgG), but no significant difference was observed due to wide deviations in antibody levels among individuals rather than groups (data not shown). In contrast, IgA antibody responses to these antigens were equal or tended to be higher in RA and SLE compared to normal controls (Fig. 6b). Accordingly, SLE shares a similar IgG and IgA antibody response pattern to those of RA, but was differentiated by higher IgA responses to LPS (Fig. 6b). On the other hand, Crohn's disease was distinguished from RA and SLE based on significantly high IgG antibody responses to *E. coli*, LPS and Pg-LPS as shown in Fig. 6a.

As a consequence of lower IgG and higher IgA antibody responses, the IgA/IgG anti-*E. coli* antibody ratios were significantly higher in G1 ($p > 0.01$), G2 ($p > 0.01$) and G3 ($p > 0.01$) of RA and SLE ($p > 0.05$) as well as the IgA/IgG anti-LPS antibody ratios in G1 ($p > 0.05$) and G2 ($p > 0.05$) of RA and SLE ($p > 0.01$) as shown in Fig. 6c. Contrary, no significant high IgA/IgG ratio was observed in Crohn's disease, in which IgG antibody responses to all three antigens were concurrently higher than those of normal controls, RA and SLE, except for the IgG antibody respose to PG-PS.

4. Discussion

False positive antibody studies encountered in the assay of antibodies in human sera are a too common of an occurrence and are not restricted to any one particular type of antigen. If proper precautions are not taken in the development of an assay and interpreting its results, the outcome can be most unfortunate. The literature is replete with such examples. This problem appears more common in the study of human sera where lower dilutions are used to enhance assay sensitivity. Nevertheless, strong false positive reactions can still be seen even with sera dilutions of 1/2000 or greater. Without being aware of the intense false positive reaction caused by the sample itself and other non-specific reactions, antibodies against a variety of antigens were assayed in human sera at low dilution as low as 1/20-1/200, as described later.

In this study, we re-confirmed that none of the conventional ELISA buffers are capable of blocking the significantly high BG noise reaction caused by hydrophobic binding of immunoglobulin components in human sera to plastic surfaces (Figs. 1 and 2). Moreover, conventional buffers were ineffective compared to ChonBlock™ at reducing false positive (Fig. 3a–i & b–i) and negative reactions (Fig. 3a–ii and b–ii) in anti-CCP and anti-*E. coli* antibody assays. In addition, the new buffer effectively eliminates the false positive reaction caused by a common blocking agent, bovine serum albumin (BSA), as well as negative reactions due to denaturing detergents such as SDS, and inhibiting antibodies in heterologous serum supplemented to sample dilution buffers as shown in Fig. 3a–iii and b–iii. Indeed, the data show that the new buffer is applicable for assaying antibodies against a wide variety of antigens including environmental agents and dietary proteins such as *E. coli*, LPS, Pg-LPS, BSA and OVA and autologous components such as CCP and collagen.

Using the new buffer system for this preliminary study, the data show that IgG antibody responses to *E. coli* and LPS are significantly lower or tend to be lower in patients with RA and SLE compared to normal controls (Fig. 6a, b & c), and as a consequence, the IgA/IgG antibody ratios are significantly higher in these patients (Fig. 6c). It cannot be ruled out that there is a possibility that steroids prescribed for these patients suppressed the IgG antibody responses; however, it is unlikely that steroids specifically affected the IgG antibody response without affecting the IgA antibody response. One possible explanation for the higher IgA/IgG antibody ratios in RA and SLE is that these patients might be chronically exposed to excess amounts of these potential environmental pathogenic agents. This may be a consequence of an imbalance of enteromicrobes in the gastrointestinal tract. In contrast, Crohn's disease was characterized by high IgG antibody responses not only to *E coli* and LPS, but also to Pg-LPS (Fig. 6a), suggesting that their immune systems are systemically and excessively stimulated by these antigens, which have penetrated into the body due to leakage of the mucosal barrier. Although it has been suggested that Crohn's disease may be linked to periodontal disease, because of the high IgG antibody responses to *P. gingivalis* (Habashneh et al., 2012), our data show that Crohn's disease patients have high antibody titers against all the antigens tested (Fig. 6a) including Pg-LPS, suggesting that excess amounts of Pg-LPS as well as other antigens are simply translocated through the intestinal mucosal membrane due to poor gut barrier function in these patients. Similarly, an association of periodontitis and RA has also been considered (Mikuls et al., 2009), although it still remains uncertain whether there is an etiological link or simply represents statistical coincidence (Farquharson et al., 2012). However, our data suggest that periodontitis may not be linked to RA, since no difference in IgG and IgA antibody responses to Pg-LPS between normal and RA was observed (Fig. 6a & b). These significant differences in immune responses to potential pathogenic agents between RA and Crohn's disease suggest pathogenic mechanisms and potential pathogens involved in RA and Crohn's disease may differ.

Notably, our observations in this study are not in agreement with past studies, which commonly concluded that antibody levels were higher in disease groups compared to normal controls; for example, antibodies against *E. coli*, *Klebsiella pneumoniae* nitrogenase, *Proteus mirabilis*, and *Serratia marcescens* (Aoki et al., 1996; Rashid et al., 2004; Zapata-Quintanilla et al., 2006; Hitchon et al., 2010), LPS (Maes et al., 2011; Arabski et al., 2012), *P. gingivalis* (Mikuls et al., 2009), glucose-6-phosphate isomerase (GPI) (Schaller et al., 2005), collagen (Collier et al., 1984; Burkhardt et al., 2006; Nandakumar et al., 2008), human HLA DR and B27 molecules (Takeuchi et al., 1990; Wilson et al., 1995; Ringrose, 1999), and citrulline (Schellekens et al., 1998; Pérez et al., 2006), *E. coli* (Newkirk et al., 2005), heat shock proteins (Komiya et al., 2011) and advanced glycation end products (AGEs) (Ligier et al., 1998). In these studies, several factors apparently contributed to the ELISA results: 1) the low dilution of test sera, 2) the buffer systems, which are not capable of blocking the most intense false positive BG noise reaction caused by the sample itself, and 3) the omission of antigen non-coated control wells in the mapping of ELISA plates to determine the unique BG noise values of individual samples.

These pioneer investigators studied antibodies against these potential pathogenic antigens with great foresight; however, the differences in our ELISA results are apparently attributed to the ability of ChonBlock™ to eliminate the aforementioned confounding factors in ELISA.

Notwithstanding, the data in this study indicate that lowered immune responses to potential pathogenic environmental agents may be the fundamental disorder in autoimmune diseases. In fact, this idea is supported by numerous studies in experimental animal models. For example, HLA-B7 transgenic mice (Reháková et al., 2000) and rats (Taurog et al., 1994) do not develop ankylosing spondylitis under germ free conditions. Similarly, GPI specific T-cell transgenic K/BxN mice do not develop arthritis under germ free conditions (Ivanov et al., 2009). Importantly, these transgenic rodents develop severe arthritis when they are transferred to non-SPF conditions or inoculated with commensal bacteria, indicating that even commensal bacterium and their toxins can be pathogenic in these animals, whose immune function is not fully functional, because of the absence of commensal bacteria, which promote the development of a mature immune system.

Actually, it has long been considered that environmental factors such as commensal bacteria and their toxins may contribute to the pathogenesis of autoimmune disease. For example, the imbalance of intestinal bacteria may be one possible etiopathogenic or aggravating factor in RA (Peltonen et al., 1994, 1997; Kjeldsen-Kragh, 1999; Tlaskalová-Hogenová et al., 2004; Edwards, 2008; Vaahtovuo et al., 2008) and inflammatory bowel disease (IBD) (Lucke et al., 2006; Frank et al., 2007; Nell et al., 2010). In addition to bacteria, the pathogenic effects of bacteria toxins produced by enteromicrobes, such as LPS, have been clearly demonstrated in a variety of animal models such as collagen-induced arthritis (Yoshino et al., 1999) and collagen antibody-induced arthritis (Terato et al., 1995), diabetes (Cani et al., 2007; Nymark et al., 2009), experimental autoimmune encephalomyelitis (EAE) (Nogai et al., 2005), SLE-nephritis (Hang et al., 1983; Cavallo and Granholm, 1991), autoimmune hemolytic anemia (Murakami et al., 1994, 1997), and in patients with obesity (Cani et al., 2007), type I diabetes (Nymark et al., 2009) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (Maes et al., 2011).

Recently, Iwatsuki et al. (Iwatsuki et al., 2011) analyzed intestinal bacteria flora in elderly volunteers treated by oral administration of a natural milk antibody product, which contains biologically active antibodies against a wide range of pathogenic bacteria and their toxins (Kijima et al., 2009), and found significant reduction in the fecal population of pathogenic enteromicrobes such as *E. coli*, *Clostridium difficile* and *perfringens*. This approach was further applied for the treatment of patients with RA, resulting in modulated disease activities in 50% of these RA patients (Katayama et al., 2011). Together, these data support the idea that commensals and their toxins may contribute to the pathogenesis of autoimmune diseases.

To further study the influence of environmental factors on the immune system, and the potential link to autoimmune diseases, it is important to determine the differences in immune function of healthy normal individuals and patients with autoimmune diseases by assaying antibody responses to environmental agents. Finally, we hope our ELISA system not only reforms antibody studies in these fields, but also improves the accuracy of diagnostics in the medical field by eliminating false positive and negative results.

5. Conclusions

A newly developed ELISA buffer, ChonBlock™, effectively prevents all types of non-specific reactions involved in ELISA, and can be applied to studying antibodies against virtually all types of antigens. Using this new buffer system, we concluded that low IgG antibody responses to potential pathogenic environmental factors might be the fundamental disorder in autoimmune diseases. We assume that patients with autoimmune diseases might be chronically exposed to excess amounts of potential pathogenic agents at the gastrointestinal tract due to an imbalance of enteromicrobes, which may be linked to the host's low immune function. We hope this report leads future studies on autoimmune diseases to new directions.

Competing interests

KT, CD, DC and TW declare that they have received salary support from Chondex Inc. HS declares that he has no competing interests.

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