Journal of Immunological Methods 443 (2017) 1–8

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Efficient evaluation of humoral immune responses by the use of serum pools

Louise Sternbæk [a], Anette H. Draborg [a], Christoffer T. Nielsen [b], Søren Jacobsen [b], Line V. Iversen [c], Lone Troelsen [d], Elke Theander [e], Gunnar Houen [a,*]

[a] Department of Autoimmunology and Biomarkers, Statens Serum Institut, Copenhagen, Denmark
[b] Copenhagen Lupus & Vasculitis Clinic, Center for Rheumatology and Spine Disease, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark
[c] Department of Dermatology, Copenhagen University Hospital, Bispebjerg, Copenhagen, Denmark
[d] Department of Clinical Immunology, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark
[e] Department of Rheumatology, Lund University, Skåne University Hospital, Malmö, Sweden

ARTICLE INFO

Article history:
Received 19 December 2016
Received in revised form 16 January 2017
Accepted 23 January 2017
Available online 25 January 2017

Keywords:
Serum pools
ELISA
Line blot
Screening
Serology

ABSTRACT

Background: Collection and testing of individual serum samples are often used in research to gain knowledge about e.g. the humoral response against bacteria or virus. This is a valid but time-consuming method and might be a waste of valuable serum samples for inefficient research. So far, no study has considered using serum pools as a quick and efficient screening method to confirm or deny hypotheses.
Methods: We created serum pools from four different patient groups (systemic lupus erythematosus n = 85, rheumatoid arthritis n = 77, Sjögren's syndrome n = 91, systemic sclerosis n = 66) and one healthy control group (n = 67). Each serum pool was analyzed using three well-known immunoassays: enzyme-linked immunosorbent assay (ELISA), line blot, and immunofluorescence microscopy (anti-nuclear antibody (ANA) screening). The presence of Epstein-Barr virus (EBV) EA/D-, EBNA-1-, VCA p23-, and gp350-directed antibodies was used to validate serum pools as an efficient tool for further investigations by comparison to previous findings in this area.
Results: The presence of EBV EA/D-, EBNA-1-, VCA p23-, and gp350-directed antibodies in each pool was consistent within the obtained ELISA and line blot results, as increased titers of IgG against the four antigens were found in all patient serum pools and also in individual sera regarding gp350. These results correspond to previous findings on individual samples from patients with these diseases. The presence of ANAs was observed in all four patient serum pools and not in the HC pool by both line blots and immunofluorescence microscopy, which corresponds with the expectations and further corroborate the application of serum pools for screenings.
Conclusion: We developed and validated the use of serum pools that reliably and rapidly can confirm or deny hypotheses, which enables a more efficient research concentrating on the most evident factors.

1. Introduction

Systemic autoimmune diseases (SADs) are a group of connective tissue diseases with diverse, yet overlapping, symptoms (Draborg et al., 2013). The etiology behind SADs is not fully elucidated, but a number of genetic and environmental factors are known to influence the incidence of SADs. Recent findings link dysregulation of Epstein-Barr virus (EBV) with SADs development (Sestak et al., 2011; Cruz-Tapias et al., 2012; Jacob and Jacob, 2012; Draborg et al., 2012, 2013). However, this remains to be fully clarified.

The measurements of EBVs involvement in SADs widely uses blood samples to screen for the humoral and/or cell-mediated immune response, and patient samples are precious and essential in research. The standard screening tests of humoral immune responses are based on measurements of immunoglobulin (Ig) levels in blood (Janeway et al., 2001). These consists of IgM, IgA, and IgG antibody levels. Blood samples are one of the most used patient materials in research as they quickly can be collected without much distress for the person and many parameters can be measured in blood. However, to be able to confirm or deny hypotheses a large sample volume, many measurements, and much time is needed. In serological research, it is never known whether the hypotheses are valid or not, and it often requires several tests, and thereby consequently large sample volumes.

Many researchers use enzyme-linked immunosorbent assay (ELISA) and line blot kits using recombinant antigens available on the market. They are used because they provide quantitative measurements of antibodies or antigens in biological samples. Some studies showed a good

* Corresponding author at: Artillerivej 5, 2300 Copenhagen, Denmark.
E-mail address: gh@ssi.dk (G. Houen).

http://dx.doi.org/10.1016/j.jim.2017.01.006
0022-1759/

performance and good concordance of the data obtained by these assays (Schaade et al., 2001; Matheson et al., 1990).

To avoid disturbing patients and volunteers more than necessary by blood collection, we want to be able to use their blood for as many analyses as possible. Therefore, we performed the initial analyses on pools of small volumes of each serum for patients and control groups, respectively. The aim of the present study was to create representative serum pools with the ability for a quick confirmation or rejection of hypotheses, which would enable a more efficient research concentrating on the most important factors.

2. Methods

2.1. Patients, controls, and pools

All patients fulfilled internationally accepted classification criteria for the autoimmune disease investigated (Hochberg, 1997; Aletaha et al., 2010; Shiboski et al., 2012; Hoogen et al., 2013). Consents for the studies were obtained from all patients in accordance with the protocol approved by the local Scientific-Ethical Committees. Serum samples were obtained from 85 systemic lupus erythematosus (SLE) patients and 80 rheumatoid arthritis (RA) patients attending the Department of Rheumatology at Rigshospitalet, Copenhagen University Hospital in Denmark. Sera from 91 Sjögren's syndrome (SS) patients were obtained from the Department of Rheumatology, Lund University, Skåne University Hospital, Malmö, Sweden and serum from 66 systemic sclerosis (SSc) patients from the Department of Dermatology, Bispebjerg, Copenhagen University Hospital, Denmark. Serum samples from 67 apparently healthy, antinuclear antibodies (ANA) and anti-dsDNA negative, controls were obtained with consent from volunteers at Statens Serum Institut, Copenhagen, Denmark.

The clinical characteristics of the 85 SLE patients, 80 RA patients, 91 SS patients, 66 SSc patients and 67 HC used in this study are outlined Table 1. The samples from patients with each disease were used by creating a pool for each disease by mixing 15 μL of each patient sample together in one tube.

Table 1
Clinical characteristics of SLE, RA, SS, and SSc patients and HC.

SLE patients	
No. of individuals	85
Average age (years), (range)	39.9 (20–81)
Females, %	92.9
Average disease duration (years), (range)	10.8 (0–37)
Average C-reactive protein (mg/L), (range)	4.7 (0–34)
On immunosuppressive medication, %	67.1
ANA-positive, %	77.6
Anti-dsDNA positive, %	43.5
Average amount of immunoglobulin (g/L)	
IgM	1.53
IgA	2.23
IgG	9.40
RA patients	
No. of individuals	77
Average age (years), (range)	55.5 (27–78)
Females, %	76.6
Average disease duration (years), (range)	14.8 (2–41)
Anti-CCP positive, %	63.6
IgM Rheumatoid factor positive, %	70.1
IgA Rheumatoid factor positive, %	57.1
Average of total SHARP score, (range)	86.1 (0−321)
SS patients	
No. of individuals	91
Average age (years), (range)	54,8 (31–77)
Females, %	91
Average age at diagnosis (years)	55.3
ANA positive, %	95.4
Anti-SSA/B positive, %	76.2
With low C3, %	21.5
With low C4, %	16.4
SSc patients	
No. of individuals	66
Average age (years), (range)	58.8 (33–76)
Females, %	87
Average disease duration (years), (range)	12 (0–53)
ANA positive, %	94.2
Average skin score, (range)	11.3 (3−31)
Anti-Scl-70 positive, %	77.9
HC	
No. of individuals	67
Average age (years), (range)	37.4 (22–63)
Females, %	82.1

2.2. Detection of EBV-directed antibodies by enzyme-linked immunosorbent assay

TTN buffer (0.025 M Tris, 0.5% Tween 20, 0.15 M NaCl, pH 7.5, SSI Diagnostica, Copenhagen, Denmark) was used for dilution, washing and blocking of samples and secondary antibodies. All incubations were conducted in volumes of 100 μL per well for diluted samples, secondary antibodies, and enzyme substrate, including both coated and non-coated wells and placed on a shaking table at room temperature (RT). All washes were performed with 200 μL per well three times each for 1 min to remove unbound protein. Carbonate buffer (50 mM sodium carbonate, pH 9.6, SSI Diagnostica, Copenhagen, Denmark) was used for coating PolySorp microtiter plates (Fischer Scientific Biotecline, Roskilde, Denmark) with recombinant EBNA1, EA/D, VCA p23 (Prospec protein specialist, Cat. No.: EBV-271, Cat. No.: EBV-272, Cat. No.: EBV-278, respectively, Ness-Ziona, Israel), and gp350 (MyBioSource, Cat. No.: MBS1071633, San Diego, USA) at a concentration of 1 μg/mL. The plate was subsequently incubated overnight at 4 °C. Afterwards the plate was washed, followed by 30 min blocking. Sera were diluted 1:10 for a 2-fold serial dilution for detection of EBNA-1-, EA/D-, VCA p23-, and gp350-directed IgM, IgA and IgG. All diluted samples were added to coated and non-coated wells in duplicates and incubated for 1 h at RT, and the wells were washed again. Alkaline phosphatase (AP)-conjugated goat anti-human IgM, IgA, and IgG secondary antibodies (Sigma-Aldrich, St Louis, MO, USA) were diluted 1:1000 for IgA and 1:5000 for IgM and IgG and incubated for 1 h. After another three washes, AP substrate (p-nitrophenyl phosphate (ρ-NPP) 1 mg/mL; Sigma-Aldrich, St. Louis, USA) diluted in AP-substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8, SSI Diagnostica, Copenhagen, Denmark) was added to all the wells. The absorbance was measured after 30 min for IgG detection and 60 min for IgM and IgA detection with a spectrophotometer (Tecan, Männedorf, Switzerland). The plate was read with an excitation wavelength of 405 nm and a reference wavelength of 650 nm. The sample absorbance values of non-coated wells (IgM, IgA, and IgG with an average <0.05) were subtracted from coated wells after averaging the duplicates.

2.3. Detection of antibodies by line blot

2.3.1. EBV antigens

Line blot assays *recom*Line EBV (Mikrogen Diagnostic, Cat. No.: 4572, Neoried, Germany) were used for detection of IgA and IgG against six EBV antigens (EBNA-1, VCA p18, VCA p23, BZLF1, EA-R, and EA-D) in a single procedure. The assays were carried out according to the manufacturer's protocol using the supplied reagents. Serum pools of SLE, RA, SS, SSc, and HC were diluted 1:100 for IgG and 1:10 for IgA detection. Test strips were incubated in 2 mL diluted sample for 1 h. After three washing steps in washing buffer, AP-conjugated goat anti-human IgA was diluted 1:1000, whereas HRP rabbit anti-human IgG was diluted 1:100 and added and incubated for another 45 min. An enzyme-specific substrate, either TMB for HRP provided by the manufacturer or BCIP/NBT (Sigma-Aldrich, St. Louis, USA) for AP, diluted in distilled water was added for 8 min for the reaction to occur. After washing in distilled water, the strips dried for 2 h. If an antigen-antibody reaction had occurred, a dark band appeared on the corresponding point on the strip.