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Analysis of complex autoantibody repertoires by surface-enhanced laser desorption/ionization-time of flight mass spectrometry

Normal sera contain a large number of naturally occurring autoantibodies which can mask important disease-associated ones. Western blotting has evolved as the most important tool to demonstrate autoantibodies in autoimmune diseases, because of its ability to simultaneous screening for a wide spectrum of different antigens. In previous studies we have shown the diagnostic potential of the analysis of autoantibodies in autoimmune diseases by means of multivariate statistics and artificial neural networks. However, the Western blotting procedure remains very time-consuming and is also limited in sensitivity. Therefore, we used an on-chip approach for the analysis of autoantibodies. This ProteinChip system uses ProteinChip arrays and SELDI-TOF MS (surface-enhanced laser desorption/ionization-time of flight mass spectrometry) technology for capturing, detection, and analysis of proteins without labelling or without the need of chemical modification. The microscale design of the arrays allows the analysis of very small quantities of proteins. In the present study, we used arrays with biologically activated surfaces that permit antibody capture studies. Protein-A-Chips were incubated with sera of patients ($n = 12$). After washing, the chips were incubated with a complex solution of autoantigens and subsequently washed again. If the Protein-A bound autoantibodies recognized their antigens, these proteins could be separated by their molecular masses and were to be detected by mass spectrometry. Previous studies using monoclonal antibodies have demonstrated that the detection limit is in the attomole level. Furthermore, all sera were analyzed by conventional Western blotting for direct comparison. In the present study, we have shown complex on-chip antibody-antigen reactions. At higher molecular weights (>30 kDa) the detection sensitivity of this on-chip method was comparable to conventional Western blotting. At lower molecular mass, the Western blot technique is easily exceeded by the on-chip method. Considering that this on-chip procedure is quite easy to use, is much less time-consuming than Western blotting, and is much more sensitive at least in the low molecular weight range, the SELDI-TOF technology is a very promising approach for the screening of autoantibodies in autoimmune diseases. Due to its versatility, this on-chip technology could allow the large-scale screening for complex autoantibody distributions for diagnostic purposes and early detection of autoimmune diseases might be possible.

Keywords: Autoantibodies / Autoimmune disease / Ocular antigens / Surface-enhanced laser desorption/ionization-time of flight mass spectrometry PRO 0375

1 Introduction

Western blotting has surfaced as a powerful tool for detecting specific autoantibodies in autoimmune diseases. Complicating the straightforward identification of

pathogenically relevant antigens, however, is that normal sera contain large amounts of natural antibodies which manifest themselves in complex staining patterns [1, 2]. Thus, it can complicate the differentiation of disease-associated autoantibodies from the complex background of "autoimmune noise" (*i.e.* naturally occurring autoantibodies).

Therefore, we recently developed a new technique where the antigen-antibody reactions were analyzed by Western blotting followed by digital image analysis [3–5]. Based on the densitographs of each Western blot, multivariate statistical techniques and artificial neural networks were used to detect differences in the distribution of antibodies

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Abbreviation: SELDI-TOF, surface-enhanced laser desorption/ionization-time of flight

against autoantigens. Recently, this technique has been successfully used in myasthenia gravis, Graves' disease and experimental uveitis [6–10]. These techniques attempt to differentiate between healthy people and autoimmune patients, considering the whole complex antibody pattern of each patient. After recognizing these patterns from known samples, it can be calculated to which clinical group the autoantibody pattern of an unknown patient reveals the highest similarity. However, the Western blotting technique is very time-consuming and limited in sensitivity.

We therefore used an on-chip approach for the analysis of autoantibodies. Surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS; Ciphergen, Fremont, CA, USA) [11] separates proteins on different solid-phase chromatographic surfaces (ProteinChip) and the proteins are subsequently ionized and detected by TOF-MS (time of flight-mass spectrometry). The ProteinChip technology is able to analyze protein profiles from a variety of complex biological materials, *e.g.*, serum, blood, tears, plasma, urine and cell lysates. This fast novel technology requires far less material compared to conventional 1- or 2-DE, and has a higher reproducibility. In the low mass range, proteins can be analyzed in the femtomole range. The search for biomarkers using SELDI-TOF can directly be used for diagnosis for diseases, *e.g.*, prostate, ovarian and bladder carcinoma [12–16]. Furthermore, the ProteinChip technology can be used for many more purposes than protein profiling. Depending on the chromatographic surfaces used in the approach, many different separations of the protein mixture become possible. Additionally, antibody capture studies can be performed. Using activated biological surfaces, antibodies can be bound to the surface.

If the antibody bound to the chip surface recognizes their antigens (proteins), then these proteins can be separated by their molecular mass and can be detected by TOF-MS. Using purified monoclonal antibodies, it could be demonstrated that detection limits can be at the attomole level [17].

In previous studies ProteinChip surfaces have only been used to detect purified monoclonal antibodies. In the present study, we attempt to detect and analyze complex mixtures of natural autoantibodies in human sera by means of SELDI-TOF and compare the results to those obtained by conventional Western blotting.

2 Materials and methods

Blood samples were taken from volunteers and centrifuged at 1000 *g* for 10 min. The serum was stored at –23°C for later examination. Ocular antigens were pre-

pared and homogenized in sample buffer (1 M Tris, pH 7.5; 10% SDS; DTT) and centrifuged at 15 000 rpm for 1 h. The samples were cooked and centrifuged several times afterwards. The pellet was discarded and the supernatant was stored for later analysis.

2.1 Western blotting

The antigen extracts were used for 13.5% SDS-PAGE using MultiGel-Long (Biometra, Goettingen, Germany). After electrophoresis the gels were transferred onto nitrocellulose membrane Protran BA 83 (Schleicher and Schuell, Dassel, Germany) by using a Semi-Dry Blotter (Biometra) for 1 h. The blots were blocked with buffer (5% non-fast dried milk in PBS for 1 h. The nitrocellulose was cut into strips and one strip *per* patient was used. The strips were incubated overnight with patients' sera (1:25 dilution, 1000 µL *per* strip). After washing the strips with TBS, they were incubated with secondary antibody: peroxidase-conjugated Immuno Pure goat antihuman IgG (H+L) (diluted 1:500, Pierce, Rockford, IL, USA) for 1 h. After several washing steps the bands were developed by 0.05% 4-chloro-naphthol (Sigma, Munich, Germany) with 0.015% hydrogen peroxide in 20% methanol in TBS for 20 min. Molecular weights were estimated for each band based on the distance migrated for ten known molecular weight standards (BenchMark, Invitrogen, Karlsruhe, Germany). Digital image analysis and evaluation of Western blots was performed by BioDocAnalyze (Biometra), which created densitometry data of the blots showing the grey-intensity values *versus* relative mobility (Rf) values.

2.2 On-chip method

In this experiment ProteinChip arrays were used for detection of antigen-antibody reactions. Analysis was performed by means of SELDI-TOF MS technology (Ciphergen Biosystems, Fremont, CA, USA). We chose a carbonyl diimidazole PS10 chip (Ciphergen Biosystems) with eight spots (A to H).

At first each spot on this PS10 chip was incubated with 2 µL of Protein A in a moist chamber at room temperature for 1 h. Then the spots were blocked with deactivation buffer (0.5 M ethanolamine in PBS, pH 8.0). During the next step washing buffer (0.5 Triton X-100 in PBS) was applied twice. The spots were washed with PBS afterwards. Six microliters of antibody solution, consisting of 0.5 µL patient's sera in PBS (also containing 0.05% Triton X-100), were applied. The last spot (H) served as control spot and was only incubated with PBS (no serum). The

chips were incubated in a moist chamber for one additional hour. Three microliters of bovine optic nerve supernatant were used as antigen and the arrays were incubated for another hour in a humidified chamber. The whole chip was washed twice in a falcon tube containing washing buffer (0.5% Triton X-100 in PBS) and put on a rocking platform for 5 min. After another washing step with nanopure water (Roth, Karlsruhe, Germany) 0.7 μ L sinapinic acid matrix (SPA) were applied on each spot, the chip was then air-dried and measured by the SELDI-TOF instrument (Fig. 1).

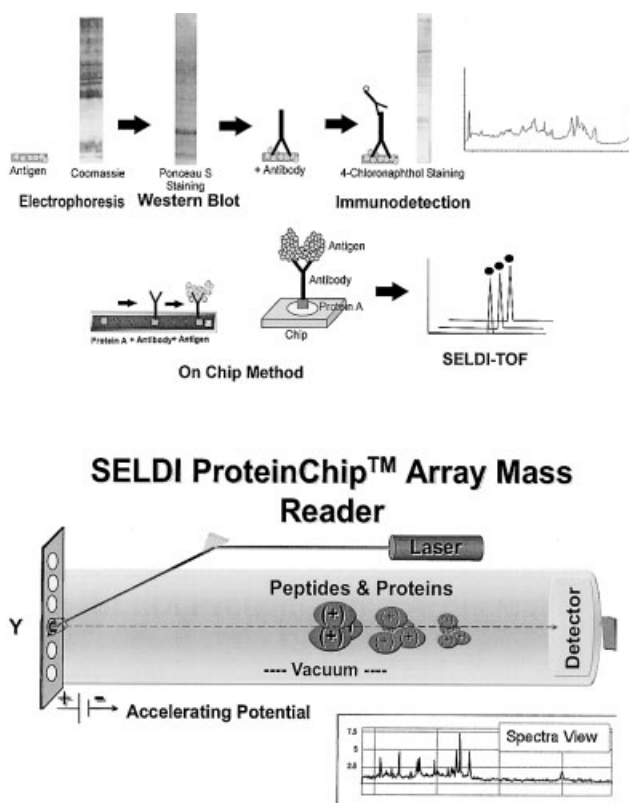


Figure 1. Western Blot: Ocular antigens were used for 13.5% SDS-PAGE. After electrophoresis the gel was transferred onto a nitrocellulose membrane by Western blotting and incubated overnight with patients' sera. After incubation with the secondary antibody (goat anti-human IgG (H+L), the bands were visualized by staining with 4-chloro-1-naphthol for 20 min. For each lane, a densitograph was created by digital image analysis. SELDI-TOF: We used a carbonyl diimidazole PS10 Chip with eight spots for this analysis. At first each spot on this PS10 Chip was incubated with Protein A in a moist chamber for 1 h. Then the spots were blocked with deactivation buffer, washing buffer, and PBS. The antibody (patient sera in PBS) was applied and chips were incubated in a moist chamber for 1 h. Ocular antigens were applied and chips were incubated for another hour. After several washing steps the matrix (sinapinic acid) was applied and the chips air-dried.

3 Results

As in previous studies, we found complex autoantibody repertoires by means of Western blotting even in the sera of healthy subjects. Figure 2 demonstrates a photograph of a complex banding pattern and the corresponding densitographic data created by BioDocAnalyze. Furthermore, we showed very complex on-chip autoantibody-antigen reactions. Figure 3 shows the antigen-antibody

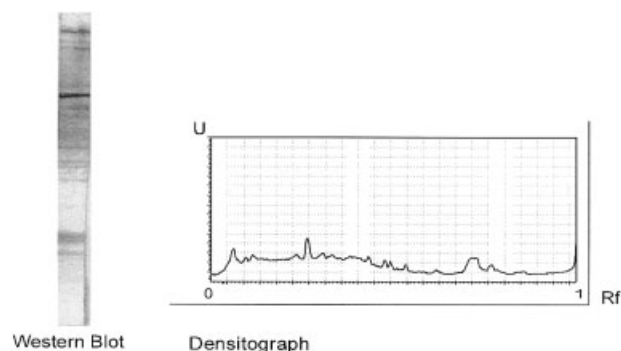


Figure 2. Right panel: Densitograph of a Western blot. Scanner units (U) are plotted vs. Rf values (relative mobility); left panel: photograph of a Western blot.

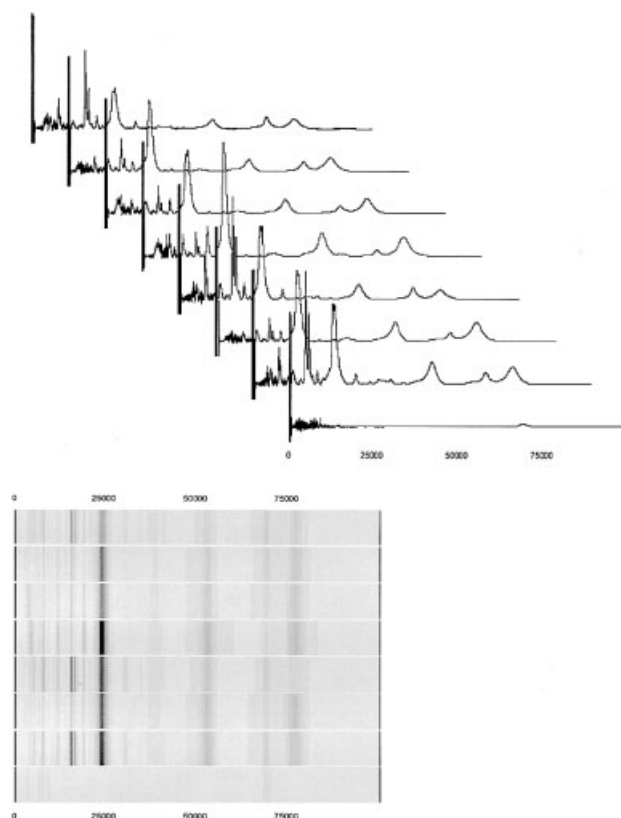


Figure 3. SELDI-TOF measurements of antigen-antibody reactions of some patients, as described in Fig. 1. In all patients' lanes, complex antibody repertoires could be detected. The bottom lane served as control (no antibody applied on the chip).

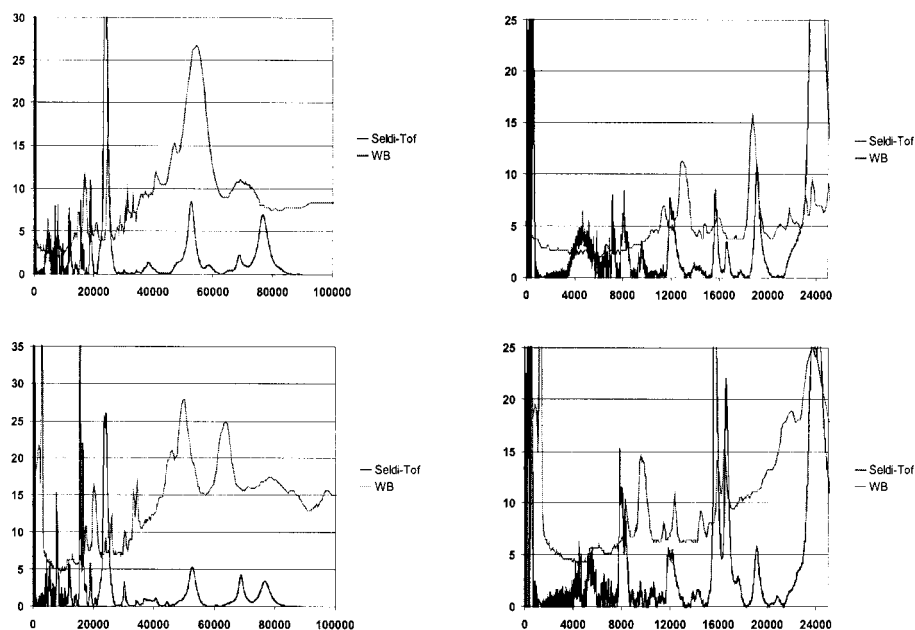


Figure 4. Comparison of antibody-antigen reactions by SELDI-TOF and antigen-antibody Western blot procedures for two different patients (A and B). For each patient, the profiles are shown in the left graphs from 0–100 kDa and in the right one from 0–25 kDa.

reactions detected by SELDI-TOF for seven different patients in the range 0–100 kDa. One lane served as control (no sera applied on the chip, only antigen mixture) and resulted in no or very small peaks. At higher molecular mass (>30 kDa) the detection sensitivity of this on-chip method was comparable to conventional Western blotting. At lower molecular mass, the Western blot technique is easily exceeded by the on-chip method (Fig. 4).

4 Discussion

SELDI-TOF MS is a very novel and promising approach combining chromatography and mass spectrometry. Although it is possible to identify proteins by digestion and conventional MALDI-TOF, the main application of this proteinchip technology is the screening for potential biomarkers, *e.g.*, to be used in the early diagnosis of disease. Furthermore, this technique allows study of biomolecular interactions and post-translational modifications on-chip, *e.g.*, for phosphorylation site mapping and glycoprotein characterization directly in the array [17].

Many proteomics studies in ophthalmology have been hampered by the need for large amounts of sample needed for the analysis by means of traditional 2-DE of proteins. Depending on the tissue or body fluid (*e.g.* tears), the available volume *per* sample is extremely limited. Thus, methods are needed that can be used with such small amounts of sample. Therefore, ProteinChip SELDI-TOF MS is a very promising tool in protein profiling, because previous studies have shown that it is very

sensitive and can even be used to analyze a mixture of just a few cells (approx. 500 cells are needed [18, 19]). Furthermore, traditional 2-DE is very time-consuming and expensive, and the reliability of this method depends largely on the experience of the investigator. The intra- and inter-investigator reliability of the electrophoretical method is often very disappointing.

In this study, we attempted to use SELDI-TOF MS to analyze complex autoantibody repertoires and compare them to conventional Western blots. We have found complex patterns in both SELDI-TOF analysis and Western blots. Surprisingly, we detected a comparable number of peaks in SELDI-TOF and Western blots at higher molecular mass. At lower molecular mass (<30 kDa) the sensitivity of SELDI-TOF far exceeded Western blotting. Furthermore, the different volumes needed for the analysis have to be considered: in Western blotting 25–50 μ L of sera were used; in SELDI-TOF just 0.5 μ L were sufficient to perform the autoantibody measurements.

Additionally, this new approach offers several more advantages compared to traditional Western-blotting such as ease-of-use, strong time-saving, and excellent reproducibility.

The Western blot procedure takes at least 2–3 d to get the results as shown above. Using SELDI-TOF, one can obtain results within approx. 2–3 h. Most of this time is due to the separation on the chip and subsequent washing procedures; the measurement itself is done in approx. 10 min/8 samples.

5 Concluding remarks

However, these investigations represent a pilot study on a new technology and method. The results have to be confirmed in further studies using a larger sample size. Furthermore, the method could perhaps be enhanced by using bioactivated chip surfaces without Protein A, because subsequently the antibodies themselves will not show up on the mass spectrum. We have shown that the SELDI-TOF procedure is easy to use, much less time-consuming than conventional procedures, much more reproducible and sensitive at least in the low molecular weight range. Thus, ProteinChip technology is a very promising approach for the analysis of autoantibody repertoires in autoimmune diseases. Considering the ease-of-use and the very short time taken to perform the analysis, SELDI-TOF could allow large-scale screening for autoantibodies for diagnostic purposes.

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6 References

- [1] Avrameas, S., *Immunol. Today* 1991, 12, 154–159.
- [2] Zimmermann, C. W., Eblen, F., Herzau, V., Reinauer, K. M., *Graefes Arch. Clin. Exp. Ophthalmol.* 1989, 227, 521–525.
- [3] Grus, F. H., Augustin, A. J., Evangelou, N. G., Toth-Sagi, K., *Eur. J. Ophthalmol.* 1998, 8, 90–97.
- [4] Grus, F. H., Augustin, A. J., *Electrophoresis* 1999, 20, 875–880.
- [5] Grus, F. H., Augustin, A. J., Toth-Sagi, K., Evangelou, N., Schmitz, T., *Invest. Ophthalmol. Vis. Sci.* 1998, 39, 536.
- [6] Zimmermann, C. W., Grus, F. H., Dux, R., *Electrophoresis* 1995, 16, 941–947.
- [7] Grus, F. H., Augustin, A. J., Toth-Sagi, K., Koch, F., *Adv. Ther.* 1997, 14, 8–13.
- [8] Grus, F. H., Augustin, A. J., Koch, F., Zimmermann, C. W., Lutz, J., *Adv. Ther.* 1996, 13, 203.
- [9] Wendtland, J. T., Grus, F. H., Augustin, A. J., Hansen, B. H., Singer, H. S., *J. Neuroimmunol.* 2001, 119, 106–113.
- [10] Dziewas, R., Kis, B., Grus, F. H., Zimmermann, C. W., *J. Neuroimmunol.* 2001, 119, 287–296.
- [11] Hutchens, T. W., Yip, T. T., *Rapid Commun. Mass Spectrom.* 1993, 7, 576–580.
- [12] Ornstein, D. K., Gillespie, J. W., Paweletz, C. P., Duray, P. H. *et al.*, *Electrophoresis* 2000, 11, 2235–2242.
- [13] Wright, G. L. J., Cazares, L. H., Leung, S. M., Nasim, S. *et al.*, *Prostate Cancer Prostatic Dis.* 2002, 2, 264–276.
- [14] Paweletz, C. P., Gillespie, J. W., Ornstein, D. K., Simone, N. L. *et al.*, *Drug Dev. Res.* 2000, 49, 34–42.
- [15] Hampel, D. J., Sansome, C., Sha, M., Brodsky, S. *et al.*, *J. Am. Soc. Nephrol.* 2001, 12, 1026–1035.
- [16] Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J. *et al.*, *Lancet* 2002, 359, 572–577.
- [17] Issaq, H. J., Veenstra, T. D., Conrads, T. P., Felschow, D., *Biochem. Biophys. Res. Commun.* 2002, 292, 587–592.
- [18] Schutze, K., Lahr, G., *Nat. Biotechnol.* 1998, 16, 737–742.
- [19] Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., Chuaqui, R. F. *et al.*, *Science* 1996, 274, 998–1001.