Auto-antibody profiling on high-density protein microarrays for biomarker discovery in the cerebrospinal-fluid.

Stéphane Roche, Yves Dauvilliers, Laurent Tiers, Carine Couderc, Marie-Thérèse Piva, Monique Provansal, Audrey Gabelle, Sylvain Lehmann

Technical note

Autoantibody profiling on high-density protein microarrays for biomarker discovery in the cerebrospinal fluid

Stéphane Roche [a,b], Yves Dauvilliers [c,d,e], Laurent Tiers [b], Carine Couderc [b], Marie-Thérèse Piva [a,d], Monique Provansal [b,d], Audrey Gabelle [b,c], Sylvain Lehmann [a,b,d,*]

[a] CHU Montpellier, Laboratoire de Biochimie, Hôpital St. Eloi, Montpellier, F-34000, France
[b] CNRS, Institut de Génétique Humaine UPR1142, Montpellier, F-34000, France
[c] CHU Montpellier, Service de Neurologie, Hôpital Gui de Chauliac, Montpellier, F-34000, France
[d] Université Montpellier 1, F-34000, France
[e] Inserm U888, Montpellier, France

ARTICLE INFO

Article history:
Received 1 April 2008
Received in revised form 27 June 2008
Accepted 1 July 2008
Available online xxxx

Keywords:
Autoantibody
Microarrays
Proteomics
Cerebrospinal fluid
Neurological disorders

ABSTRACT

Detection of autoantibodies, which are involved in tissue injury and/or the reporters from the immune system of various pathologic events, has an important potential for diagnosis, prognosis, disease staging and treatment selection. This explains the interest for new proteomics technologies, such as the high-density protein microarray used here, that allow a high-throughput, multiplexed and sensitive detection of specific autoantibodies. So far, most of the research has been performed on blood. In this note, we focus on the cerebrospinal fluid in an attempt to address autoimmune events associated with neurological disorders. Importantly, the cerebrospinal fluid is quite different from the blood in terms of protein composition and concentration. We had therefore to adapt the available blood protocols. We present here the result of our optimization that will be useful to carry out full scale immunological studies of the cerebrospinal fluid using high-density protein microarrays.

1. Introduction

Autoantibodies are useful biomarkers for the diagnosis of autoimmune diseases (for review see (Bizzaro, 2007)). In many cases, they are directly involved in tissue injury, and therefore represent interesting therapeutic targets. Alternatively, they could be the reporters from the immune system of various pathologic events, including malignant transformation (Tan and Zhang, 2008). Conventional quantitative immunological methods are often used to detect autoantibodies linked to specific organ-specific autoimmune diseases. However, new proteomics approaches have been recently employed to perform autoantibody multiplex measures, and large screening for biomarkers discovery programs (Tozzoli, 2008). High-density protein microarray is one of the high-throughput technologies that allows for the rapid identification of autoantibodies. In fact, these arrays can be spotted with thousands of individual recombinant proteins, and used in highly multiplexed and sensitive assays to detect specific autoantibodies. They have been already used to follow the blood immune response profile through which disease-specific antibodies might be detected leading to new diagnosis, prognosis and personalized medicine (Mattoon et al., 2005).

Here, we decided to adapt this microarray technology to the cerebrospinal fluid (CSF). The rationale was to be able to address autoimmune events possibly associated with neurological disorders. Although the central nervous system is partially protected from circulating antibodies by the blood–brain barrier, autoimmunity has been involved with different degrees of certitude in many pathological situations such as: multiple sclerosis, paraneoplastic encephalitis, cerebral manifestations of connective disorders, narcolepsy, epilepsy… In addition, as in malignant transformation, neuronal damage could also render cellular components immunogenic and trigger specific autoimmune response. The CSF is a peculiar biological fluid that has a low protein concentration, but a

* Corresponding author. CHU Montpellier, Laboratoire de Biochimie, Hôpital St. Eloi, Montpellier, F-34000, France. Tel./fax: +33 4 99 61 99 31.
E-mail address: Sylvain.Lehmann@igh.cnrs.fr (S. Lehmann).

high diversity and an important variability upon pathologic processes. It was therefore important to provide to the scientific community technical information that will be useful to initiate full scale immunological studies of the CSF using high-density protein microarrays.

2. Materials and methods

2.1. Materials

Invitrogen ProtoArray® 4.0 used here are high-density protein microarrays spotted with more than 8000 recombinant human proteins. Each human open reading frame (ORF) is expressed as an N-terminal GST fusion protein, purified, and printed in duplicate on the nitrocellulose-coated glass slide.

Anonymised CSF samples originate from the methodological biobank of the CHU laboratory (registration number from the "Agence française de sécurité sanitaire des produits de santé" pending). Consequently, no clinical information other than the general diagnosis (i.e. Alzheimer's disease, narcolepsy and multiple sclerosis) was available for this technical study. These samples were colleted using strict preanalytical guidelines as follows. CSF was directly collected in polypropylene tubes, centrifuged (10 min, 1000 *g*) in the next 4 h, aliquoted by 0.5 ml in Eppendorf and stored at −80 °C. To test multiple experimental conditions, a pool of CSF made from 20 different non hemorrhagic or xanthochromic samples was used.

2.2. General procedure for probing the microarray with CSF

For the purpose of this study, we used both control slides that are spotted with a small number of proteins and controls (Fig. 1), and complete slide for the analysis of individual CSF (Fig. 2). The description of the arrays with the position and the identification of the different spots are accessible on the Invitrogen website (www.invitrogen.com/protoarray). To use these microarrays with CSF, we first adapted the probing protocol as follows:

- Protein arrays were positioned in plastic rectangular chambers (Greiner, 96077308) and non specific sites

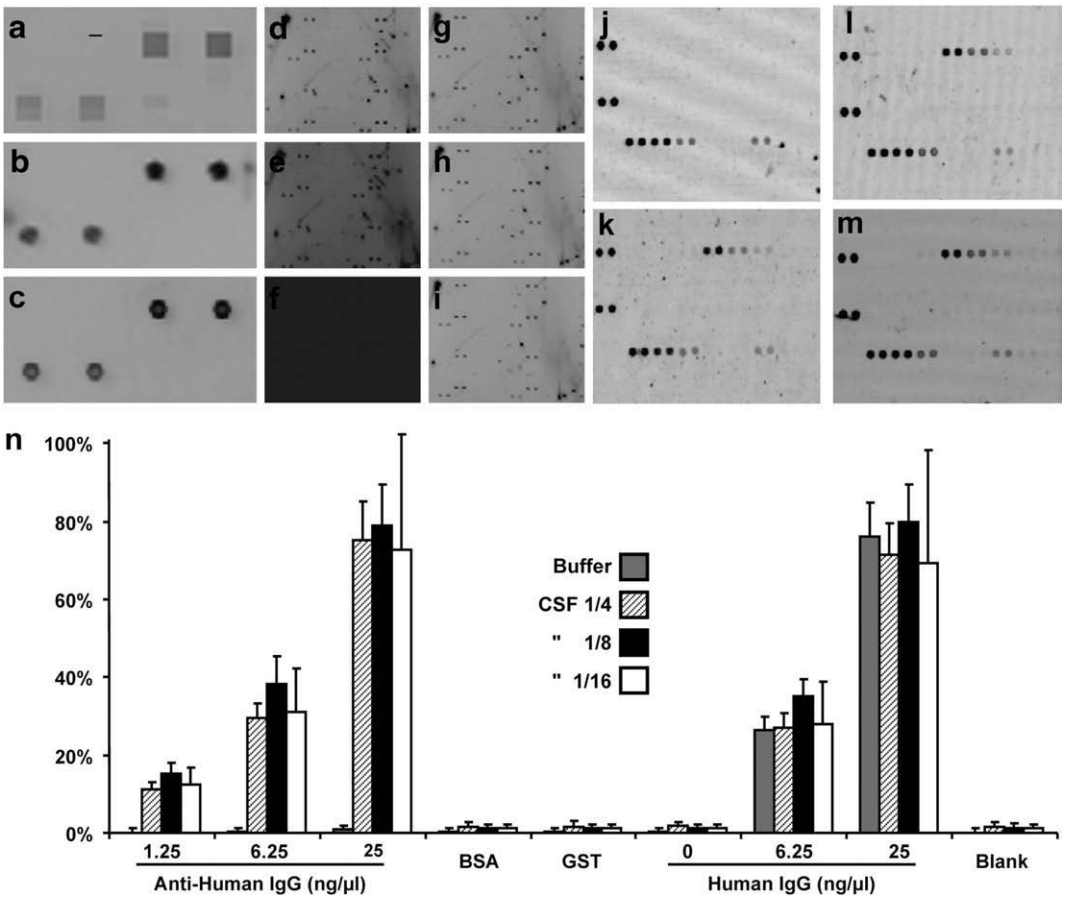

Fig. 1. Optimization of the analysis protocol. To optimize spot detection on the Typhoon Trio+ scanner, resolution of 100 µm (a), 25 µm (b) and 10 µm (c) were initially tested on control slides. Photomultiplier (PMT) power (amplification) with 600 (d), 800 (e) and 1000 V (f), as well as different sensitivity: low (g), medium (h) and high (i) were also evaluated. In the end, a resolution of 10 µm with an amplification of 600 V and a sensitivity on "high" were the selected settings. To select the condition of analysis, arrays were probed with the buffer only (j) or with the CSF pool diluted at 1/4 (l), 1/8 (k) and 1/16 (m). Quantitative results (as a % of the maximum fluorescent value) are presented in the graph (n) as follows: at the left hand side, 3 spots with different concentrations of anti-human IgG (to capture the IgG present in the liquid), empty spot (blank) and spots loaded with BSA and GST proteins (to evaluate the non specific bindings of IgG), then 3 concentrations of human IgG (to evaluate the secondary antibody). No significant differences were observed between the three CSF dilutions.

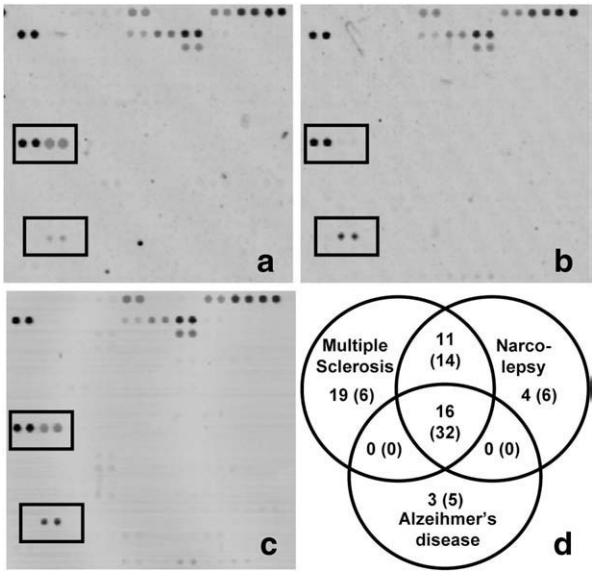

Fig. 2. Individual analysis of three CSF. Only part of the array slides is presented. CSF linked to three different diagnoses were used: narcolepsy (a), Alzheimer's disease (b) and multiple sclerosis (c). Spots were present in duplicates on the slide and corresponded to identified proteins or controls (as mentioned in Fig. 1). Some of these spot were positive in all three conditions, other only in some (see frames). The distribution of the positive spots between the three conditions is represented panel d. The number of positive spots selected using the CI *p*-value (first number) or the *z*-score (in parenthesis) are indicated in the different sections on the chart.

blocked during 1 h on ice by incubating with 1% BSA (Sigma A-8577) and 20 mM Reduced Glutathione (Sigma G-4251) in 50 mM HEPES pH 7.5, 200 mM NaCl, 0.08% Triton X-100, 25% Glycerol, 1 mM DTT.

- The buffer was removed and 500 µl of CSF eventually diluted in 1% BSA and 0.1% Tween 20 in phosphate buffer saline (Probing Buffer) were carefully put on the array and overlaid by a HybriSlip™ cover (Invitrogen H18200) in order to reduce amount of sample used.
- After 90 min of incubation on ice, the coverslip was carefully removed, and the protein arrays were rinsed four times 8 min in large volumes of Probing Buffer.
- The arrays were then incubated 90 min under slow agitation, at 4 °C, in the dark, with a goat anti-human IgG (H+L) labelled with Alexa Fluor® 647 (Invitrogen A21445) diluted in Probing Buffer at 1/2000.
- Arrays were finally washed four times 8 min with large volumes of Probing Buffer and dried in the dark 60 min before further processing.

Protein microarrays were scanned using the Amersham Typhoon Trio+ and Typhoon Scanner Control v5.0 software. To optimize spot detection different settings were tested: resolution, amplification and sensitivity (see below). Protoarray Prospector 4 Software used for microarray analysis is freely available from Invitrogen. Two statistical tools were used to detect positive spots, CI *p*-value and *z*-score. They correspond respectively to the probability that a spot is similar to a negative control, and to the spot signal minus average (for all spots) divided by the standard deviation for all spots.

3. Results and discussion

3.1. Characteristics of the CSF

Optimised protocols for using Invitrogen ProtoArray® to detect blood autoantibodies are available (see for example www.invitrogen.com/protoarray). However, to our knowledge, procedures adapted to the CSF on this type of microarrays do not exist on commercial web-sites, nor in the literature. As the serum, the CSF has an important protein diversity that results from both filtration of blood through the blood–brain barrier (80%) and brain production/secretion (20%) of peptides and proteins including immunoglobulins (Ig) (Huhmer et al., 2006). CSF normal protein concentration ranges from 0.2 to 0.4 mg/L, a value hundred-time lower than that in the blood. In pathologic conditions, blood protein concentration varies no more than 2–3 fold, whereas in the CSF, ten fold differences can be observed. In addition, common protein components were not found with the same relative amount in both fluids. For example, the percentage of total protein represented by IgG is 13% in serum and only 7% in CSF. In fact, while Ig enter the CSF via the choroid plexus, their concentrations are critical depending on their hydrodynamic radius (Reiber, 2001). For this reason, IgM concentration is much lower than in the serum where they account for 9% of total protein, but they can still be measured in different neurological diseases as reported by Reiber (1994). The search for autoantibodies in this fluid has therefore to be adapted to these CSF characteristics.

3.2. Optimization of the scanning

To scan the microarrays, Invitrogen recommends the GenePix® scanner (Molecular Devices Corporation). However, we performed our scanning using the fluorescence scanner Amersham Typhoon Trio+ that was readily available in our institute, and provided comparable results on test slide when compared to the GenePix® scanner (not shown). To optimize the use of this analyser, different resolutions, amplifications and sensitivities were tested on a CSF pool. The highest resolution (10 µm) of the scanner was selected as it provided a better definition of the spot (Fig. 1a–c), and the best average signal/noise ratio calculated for a series of spot (2.5, 2.3 and 1.8 for the 10, 25 and 100 µm resolution, respectively). As recommended by Amersham, different Photomultiplier (PMT) powers (amplification) were tested (600 V, 800 V and 1000 V, see Fig. 1d–f). The lower voltage achieved the best average signal/noise ratio on a selected series of light spots (1.72 vs. 1.37 and 1.1 for 800 V and 1000 V) and was therefore selected. Finally, the three pre-selected sensibility were tested (high, medium and low, Fig. 1g–i). The three conditions resulted in comparable results and the high sensibility which could help detect weak signals was selected.

3.3. CSF analysis

The amount of CSF necessary to probe the arrays was a crucial and limiting factor knowing that CSF samples are often small (a few mL or less), and that this fluid has hundred-time less proteins and Ig than blood. As no data were available with this fluid, dilution of 1/4, 1/8 and 1/16 of a CSF pool were

compared to an array probed with the buffer only (Fig. 1j–m). The three dilutions were comparable in terms of number of spot detected. To verify the impact of these different dilutions, we evaluated the signal on three similar series of control spots (Fig. 1n), as follows. The amount of total IgG present in the sample dilutions were in large excess, as demonstrated by the similar signal obtained on the control anti-human-IgG spots. Importantly, even the lower CSF dilution did not result in the detection of false positive, as revealed by the absence of signal on the two control proteins (bovine serum albumin — BSA and Glutathione *S*-transferase — GST) or on a empty spot (blank). The binding of the secondary antibody on spots with different amount of purified IgG was also not modified by the different quantities of CSF used. For all these data, no significant differences were observed between the different CSF dilutions. The 1/4 CSF dilution was finally chosen to increase the capacity to detect small amount autoantibodies without having significant false positive.

3.4. Protein array analysis

Using the optimized protocol and experimental conditions described above, three individual CSF belonging to confirmed cases of narcolepsy, multiple sclerosis and Alzheimer's disease, were used on the high-density protein microarrays. To detect positive spots, two statistical tools were available in the Protoarray Prospector 4 Software: CI *p*-value and *z*-score with the respective standard cut-off of 0.001 and 4. The number of spots positive for narcolepsy, multiple sclerosis and Alzheimer's disease CSF samples were for CI *p*-value: 31, 46, 19 and for *z*-score: 49, 53 and 37, respectively. The discrepancy between the two statistical tests illustrated the difficulty and the importance of the bioinformatics analysis. To be on the safe side, the company suggests, while doing a complete experiment, to consider only spots that would be positive to both tests. In our dataset, the positive spots were present in one or several samples as illustrated Fig. 2d. As auto-IgG antibodies are commonly found in the serum of patients (even with no autoimmune disease), the presence of positive spots common to the three types of samples was expected. In addition, it was also comforting that narcolepsy and multiple sclerosis, which physiopathology are though to be linked to autoimmunity (Dauvilliers and Tafti, 2006; Tozzoli, 2008), had significantly more positive spots than Alzheimer's disease. The presence of autoantibodies specific for a pathology could lead to new diagnosis procedure. However, the results obtained here have no statistical value, and have to be view only as a proof of concept leading to the realization of further experiment using a higher number of samples, in a full clinical study.

4. Concluding remarks

As reported in this work, it was possible to adapt to the CSF the screening of autoantibodies using high-density protein microarrays. Importantly, the volumes of liquid needed for the analyses were compatible with the size of clinical samples. The data obtained were also reproducible and informative. This technical adaptation will therefore be important to carry out further investigation on the possible role of autoantibodies in neurological disorders, and on the interest of their detection for diagnosis purposes.

Acknowledgements

Supported by grants from the EC FP6 program cNEUPRO, the "Institut National contre le Cancer" (INCa), the CHU of Montpellier and the CNRS.

Competing interests

For analytical evaluation, the microarrays were provided at half price by the Invitrogen company.

References

Bizzaro, N., 2007. Autoantibodies as predictors of disease: the clinical and experimental evidence. Autoimmun. Rev. 6, 325.
Dauvilliers, Y., Tafti, M., 2006. Molecular genetics and treatment of narcolepsy. Ann. Med. 38, 252.
Huhmer, A.F., Biringer, R.G., Amato, H., Fonteh, A.N., Harrington, M.G., 2006. Protein analysis in human cerebrospinal fluid: physiological aspects, current progress and future challenges. Dis. Markers 22, 3.
Mattoon, D., Michaud, G., Merkel, J., Schweitzer, B., 2005. Biomarker discovery using protein microarray technology platforms: antibody-antigen complex profiling. Expert. Rev. Proteomics 2, 879.
Reiber, H., 1994. Flow rate of cerebrospinal fluid (CSF)—a concept common to normal blood-CSF barrier function and to dysfunction in neurological diseases. J. Neurol. Sci. 122, 189.
Reiber, H., 2001. Dynamics of brain-derived proteins in cerebrospinal fluid. Clin. Chim. Acta 310, 173.
Tan, E.M., Zhang, J., 2008. Autoantibodies to tumor-associated antigens: reporters from the immune system. Immunol. Rev. 222, 328.
Tozzoli, R. (2008) Review: The diagnostic role of autoantibodies in the prediction of organ-specific autoimmune diseases. Clin Chem Lab Med.