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Antigen Microarrays for Rapid Screening of Rheumatoid Arthritis and Other Autoimmune Diseases

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INTRODUCTION

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic and extremely disabling disease, primarily characterised by extensive synovial inflammatory attack, leading to progressive autoimmune destruction of cartilage, ligaments and occasionally bone[1-4]. Approximately 580,000 patients in England and Wales suffer from this multi-factorial disease[2], costing the UK an estimated £3.8-4.75 billion per annum[4,5]. As the complexity of the disease aetiology is still relatively unidentified, the disease remains incurable[6].

Diagnosis

In 2009, NICE guidelines advocated the importance of early RA diagnosis, as Disease Modifying Anti-Rheumatic Drugs (DMARDs) significantly enhance prognosis if started early in disease progression[4]. This led to the 2010 American College of Rheumatology diagnostic criteria[7], as previous 1987 criteria depended heavily upon clinical presentation, which is equivocal in early stages[8,9]. It also relied upon detection of Rheumatoid Factor (RF)[9,10] that, despite being highly sensitive, is poorly specific. (Approximately 60% of Sjögren's Syndrome patients and 5% of healthy populations are sero-positive[3,11].) Hence, novel laboratory diagnostic techniques evolved with a greater sensitivity, specificity and ability to detect RA before irreversible joint destruction occurs [3,12].

A prime example is testing for anti-citrullinated protein antibodies (ACPA) against a synthetic peptide termed cyclic citrullinated peptide (CCP). These are well recognised serological markers of RA presenting years before symptom onset[13], and are virtually absent in healthy individuals and other rheumatic diseases[14]: this is because inflammatory processes in RA release Peptidylarginine Deiminase (PAD) enzyme into the synovium. PAD converts arginine subunits of membrane proteins into citrulline and researchers believe this post-translational modification alters protein antigenicity, triggering autoimmune processes[8].

Research is currently investigating the spectrum of citrullinated proteins involved in pathogenesis and their relationship with certain ACPA isotypes and classes/ subclasses [11,15-18].

Recent ACPA ELISAs have the ability to be, as a minimum, equal to RF sensitivity (82%) with an even greater specificity (98%)[11]. Consequently, ACPA assays have emerged as a superior diagnostic test to RF and have been incorporated into the 2010 American College of Rheumatology criteria[7,19]. However, current ELISA techniques limit the possibility of screening worldwide arthritis populations for all ACPA isotypes and specificities, due to time-scales and costing. For this reason, there is emerging potential to develop an improved technique with this capability.

Protein Microarrays

Advances in technology have brought protein microarrays to the forefront as a validated method of autoantibody detection and have so far shown very promising results[16,20,21]. They can simultaneously detect multiple autoantibodies and ACPA isotypes in a single assay, whilst maintaining similar sensitivities and specificities to current ELISA methods[22]. By aiding diagnosis and management, monitoring response to therapeutics and identifying disease subgroups or novel autoantigens[21], microarray potential in the world of immunology is far superior to current methods.

Research Proposal

The project aim is to investigate a new method of screening for autoantibodies in the serum of autoimmune patients, including RA, to aid patient categorisation.

Protein microarrays will particularly assess the significance of known autoantigens, second generation cyclic citrullinated peptides (CCP2), citrullinated proteins and their non-citrullinated counterparts. Antigens will be robotically printed onto microscope slides alongside

control proteins. The assays will screen donated patient sera for autoantibodies and, in terms of RA, the project will hope to observe ACPA in patient sera. Only a tiny volume of patient serum (less than 5µl, compared to 5ml required with ELISA21) will be required to simultaneously screen for autoimmunity against a wide range of autoantigens.

Objectives

1) To analyse protein microassay potential of diagnosing RA in early stages.
2) To determine whether protein microarrays can provide useful information in other autoimmune conditions.
3) To establish the possibility of creating biomarker profiles that relate to disease severity, course and prognosis.
4)To discover if protein microarrays have the ability to classify patients into disease subgroups.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

All chemicals and reagents used in the following experiments were purchased from SIGMA ALDRICH CHEMISTRY®, USA and stored at room temperature, unless otherwise stated. Experiments were also carried out at room temperature, unless otherwise stated.

Antigens

CCP2 was printed in both PBS and in Dimethyl Sulfoxide (DMSO) solvent to aid correct peptide formation, along with a range of citrullinated proteins and their non-citrullinated counterparts. Citrullination was performed prior to this study.

Various other antigens covering a wide range of autoimmune diseases were also printed alongside putative Chronic Obstructive Pulmonary Disease (COPD) biomarkers - as both COPD and RA are linked with smoking, it has been questioned whether COPD biomarkers act as autoantigens in RA[11](**Figure 1**).

Stock antigen samples were diluted using a 5 times stock solution of PBS-Trehalose-Tween20 buffer (0.5mls PBS, 0.5mls Trehalose and 2µl Tween20) to give 10 µl of a of 0.1µg/µl concentration. Samples with unknown stock concentrations were assumed to be at a concentration of 1mg/ml, and samples with very weak stock concentrations were not diluted. Calculations were produced to ensure the buffer used in every sample was at a 1 times concentration.

Printing Proteins

Each sample was transferred into a 384 well microtitre plate and printed onto PVDF coated slides by means of a Genomic Solutions Microgrid II 610 Arrayer with use of a silicon PETC (partially etched through channel) pin (PARALLEL SYNTHESIS TECHNOLOGIES, USA). Three triplicate spots of autoantigens were printed in each grid to monitor reproducibility. Two rows of a 2-fold serial dilution of human IgG (with the same diluent and buffer as above) were also printed per grid to serve as a positive control.

In each case, proteins were robotically printed in a 12x12 grid with each spot approximately 150 microns in diameter. Sixteen identical grids (roughly 6x6mm each) were printed onto each slide in an 8x2 array (**Figure 1**).

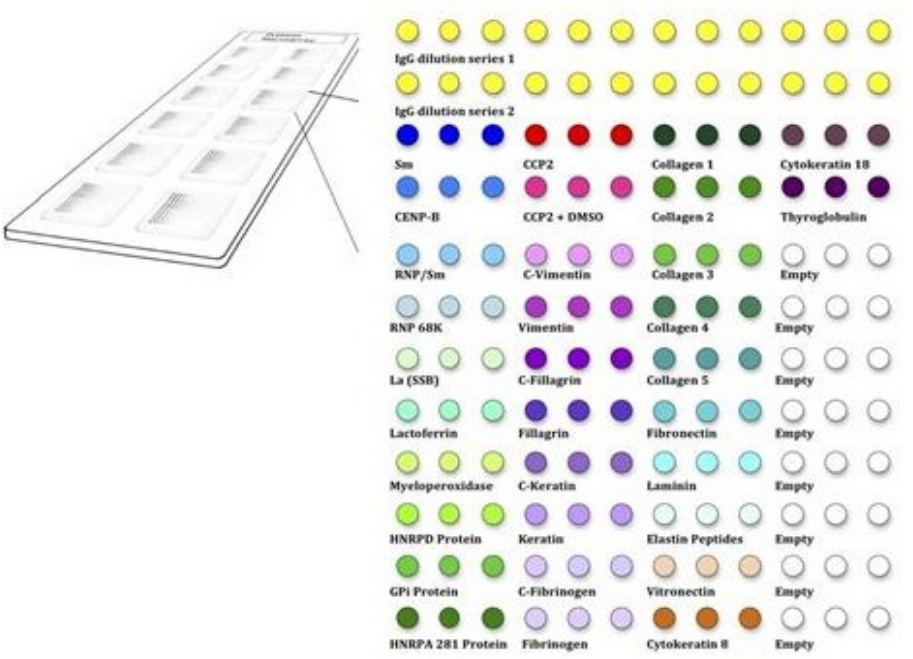

Figure 1: Autoantigens were printed in 12 X 12 squares, in the adjascent format, onto PVDF-coated slides using a microarrayer

Probing Slides

1) Slides were inserted into a Grace-BioLabs 16-well plastic slide gasket
2) One hundred micro litre (100µl) of 0.2% I-block (0.05 grams of I-block pellets dissolved in 25mls of Phosphate Buffered Saline-Tween 20[PBS-Tween]; stored in a fridge) was added to each well [NB. A 1 litre stock solution of PBS-Tween was previously prepared using 10 PBS tablets dissolved in 1 litre of distilled water and 500µl of 100% Tween20].
3) Slides were covered with cling-film and left to shake for 1 hour at room temperature
4) Each well was washed with PBS-Tween 3 times, each for 5 minutes
5) One hundred micro litre (100µl) of patient serum (1:100 with Antibody Diluent) was added to each well
6) Slides were covered with cling-film and left to shake for 1 hour
7) Each well was washed with PBS-Tween 3 times, each for 5 minutes
8) One hundred micro litre (100µl) of biotinylated anti-human IgG antibody (1:1000 in Antibody Diluent) was added to each well
9) Slides were covered with cling-film and left to shake overnight in a coldroom at 4^{o}C
10) Each well was washed with PBS-Tween 3 times, each for 5 minutes
11) One hundred micro litre (100µl) of Streptavidin-IR780 (1:5000 in PBS-Tween) was added to each well
12) Slides were covered with silver foil and left to shake for 30 minutes
13) Each well was washed with PBS-Tween 3 times, each for 5 minutes
14) Slides were briefly washed with distilled water, immediately dried with dry nitrogen gas and scanned using a a Licor Odyssey Infrared Scanner (School of Biomedical Sciences) to obtain digital images for analysis
15) Primary data acquisition (spot identification, feature and background measurements) was performed using Molecular Devices Genepix Pro V6.25 software within the Post-Genomic Technologies Facility, A floor, West Block, QMC

Patient Serum

Thirty patient sera samples were probed onto two PVDF coated slides. The samples encompassed a wide range of autoimmune diseases, including the same patient over time, whilst receiving treatment and during a flare of symptoms. Three RA, 11 SLE, three Palindromic Arthritis (PA), two Scleroderma, two Myositis, four Wegener's Granulomatosis and five Sjögren's Syndrome samples were tested (**Figure 2**). Wells 15 and 16 on one of the slides were probed with I-block instead of primary antibody to serve as control wells.

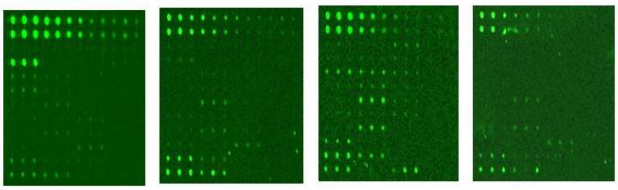

***Figure 2*: Florescent images obtained from Sera Test. Reading left to right, images obtained were from 0946 Scleroderma, 0954 SLE, 1071 Palindromic Arthritis and 0934 RA sera samples. Refer to **Figure 2** for a layout of autoantigens.**

Analysis

Graphs were created using Prism 5.04 (GraphPad Prism Inc)[23]. Comparison of sample profiles was undertaken using MeV 4.6 (Institute for Genome Research)[24]. Due to the small number of samples available, statistical analytical methods were limited. Hierarchical Clustering Algorithms using Pearson's Correlation were successfully created to determine the propensity of samples from similar disease diagnoses to cluster together.

RESULTS

IgG Dilution Series

Signals were detected consistently and as expected with the IgG dilution series, validating the techniques and methods used.

Sera Test

Of the three RA samples tested (**Figure 3**), results obtained suggested negligible autoantibody titres throughout. CCP2 peptides produced weak signals, despite two samples known to be CCP2 positive. Subsequent testing later indicated that the CCP2 peptide

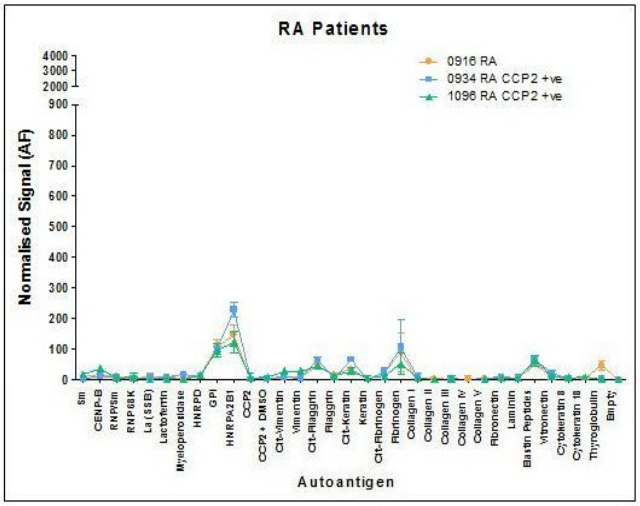

Figure 3

did not bind successfully onto the PVDF slides, limiting analysis potential. Small signals were obtained with citrullinated Filaggrin and Keratin, yet not with citrullinated Fibrinogen or Vimentin. In relation to the putative COPD biomarkers, low signals were obtained.

PA patients appeared to have similar autoantibodies to RA, but to different citrullinated proteins (Vimentin, Keratin and Fibrinogen) and with higher autoantibody titres (**Figure 4**). As PA is associated with ACPA[25], comparing Graphs A and B may suggest that PA is serologically more reactive than RA, yet similar in terms of autoantibody targets.

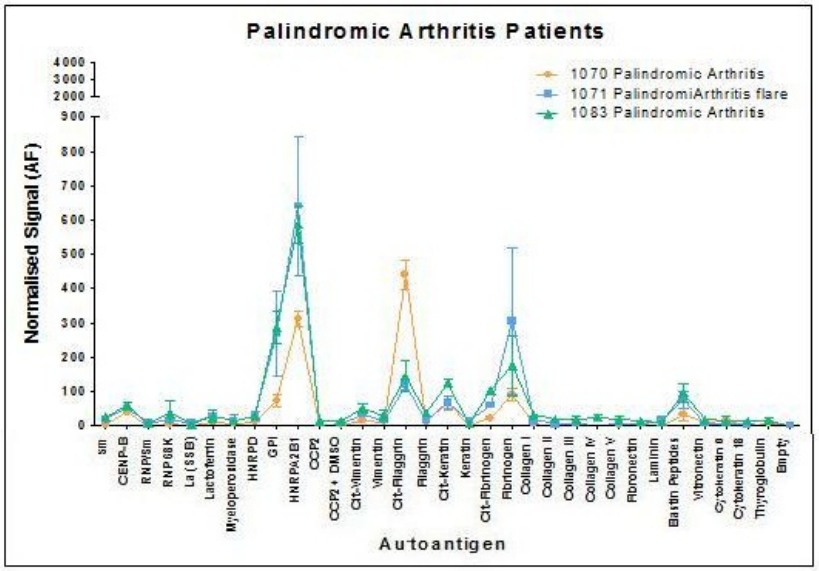

Figure 4

Autoantibody responses were largely similar between the two Scleroderma patients (**Figure 5**).

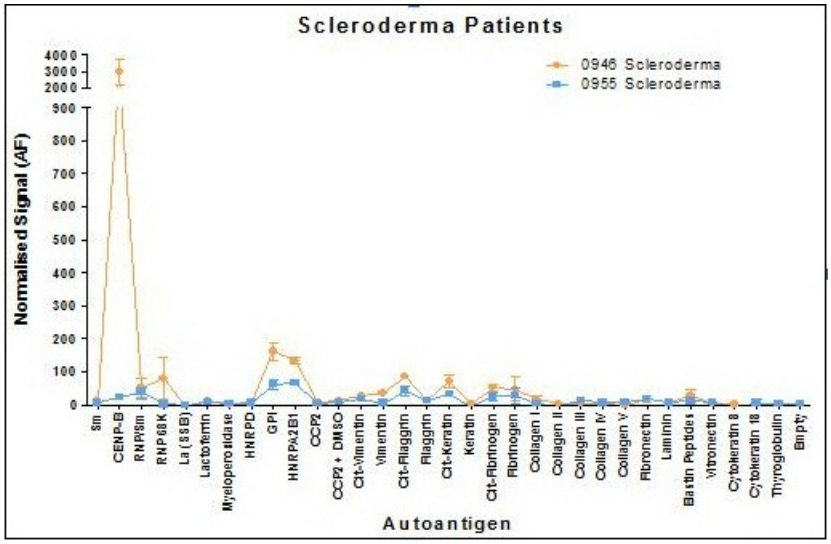

Figure 5

SLE responses (**Figure 6 and 7**) are much more varied between individual patients than all other diseases tested. As expected, RNP/Sm, RNP 68K and La(SSB) gave several high signal values [26].Samples 0903 and 0954 were taken from the same patient over time and results demonstrate a 2-fold increase in autoantibodies to Cytokeratin 8. In total, three patients with alleged inactive forms were tested: sample 1067 had relatively low signal values across the range of autoantigens, whereas the remaining two samples (0903 and 0918) had comparatively high signal values. When comparing sample 0903 with an active sample taken from the same patient (0954), it is clear that inactive signal intensities are far smaller than in the active sample. This highlights the relationship between autoantibody titre and clinical presentation in SLE.

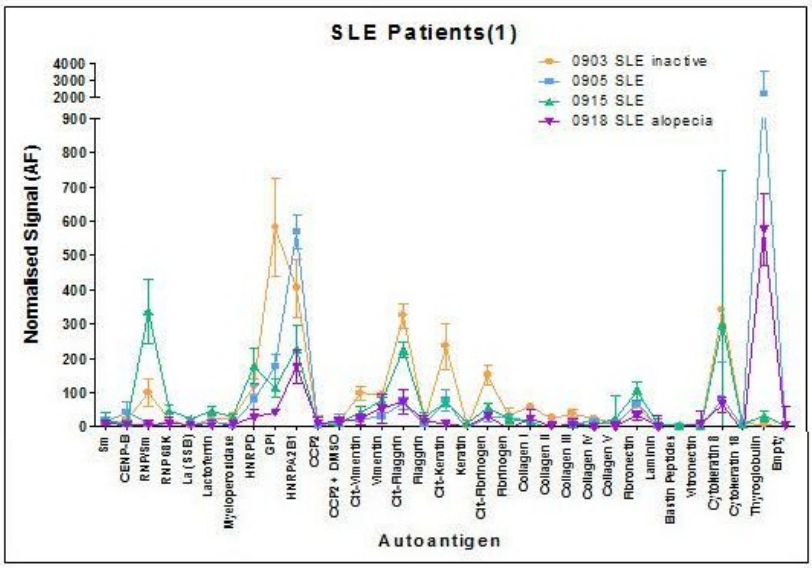

Figure 6

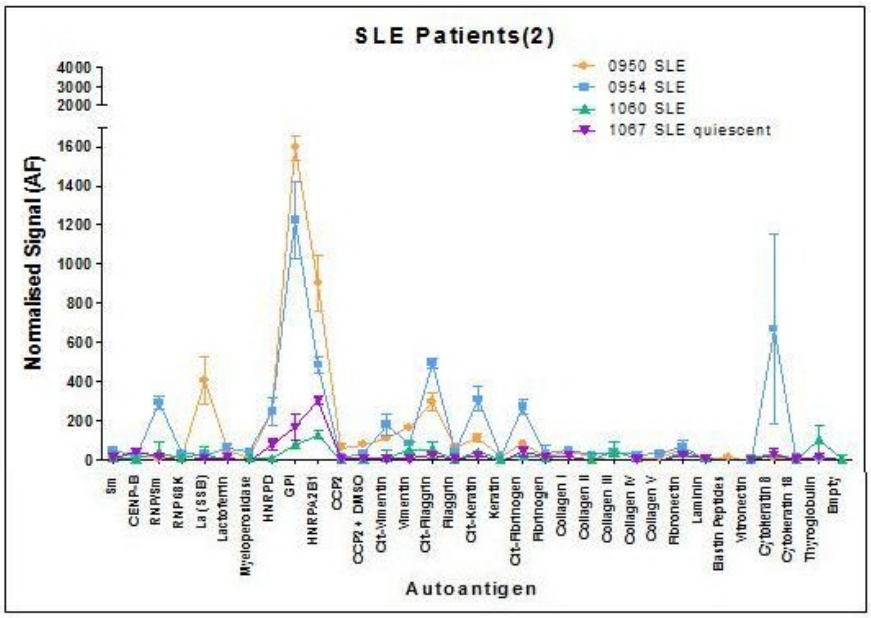

Figure 7

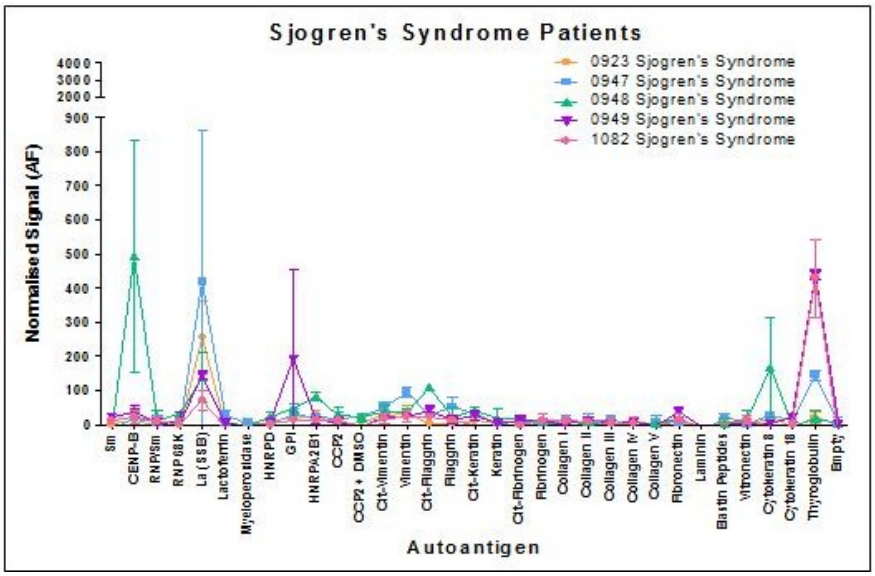

Figure 8

Sjögren's Syndrome data (**Figure 8)** also illustrates variation between individual patients. As evidence suggests[26], autoantibodies were present against both CENP-B and La(SSB), yet signals were absent against RNP/Sm or RNP/68K.

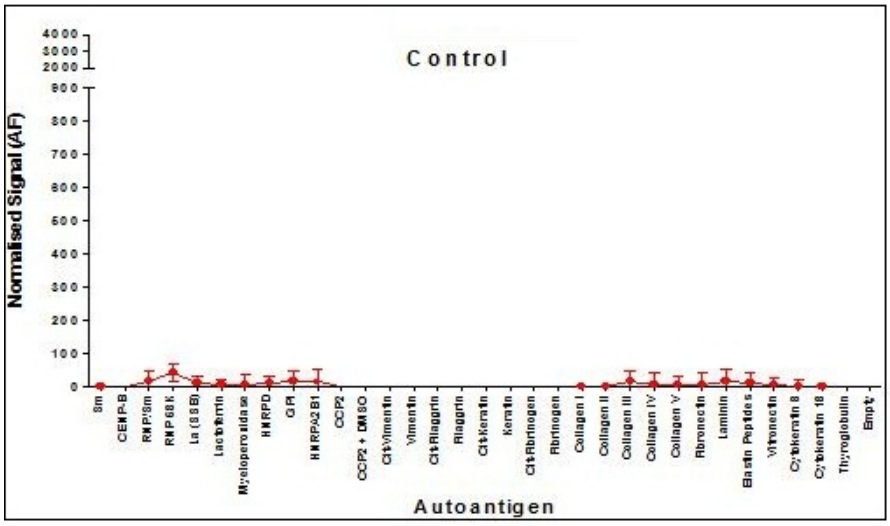

Figure 9

Figure 9 demonstrates the very small signals generated in the absence of primary antibody. Consequently, any signals above 1 standard deviation of the mean control values have been considered as positive signals.

Analyses comparing differences between several autoimmune diagnoses reveals the true potential of microarrays for diagnosis, classification and recognition of specific biomarker profiles that relate to evolution of new diseases and subsets. **Figure 10** is a cluster representation of the patterns recognised between samples. The majority of RA and PA samples cluster together, highlighting strong similarities between the autoantibody profiles of these two conditions. In terms of the autoantigens, there is a strong cluster of antibodies towards Fibrinogen and Elastin Peptides in both RA and PA samples, indicating a possible role for these antigens in the two disease pathogeneses. In addition, there appears to be two distinct clusters of SLE samples that have distinguishably different autoantibody profiles, and all of the Sjögren's Syndrome samples clustered together.

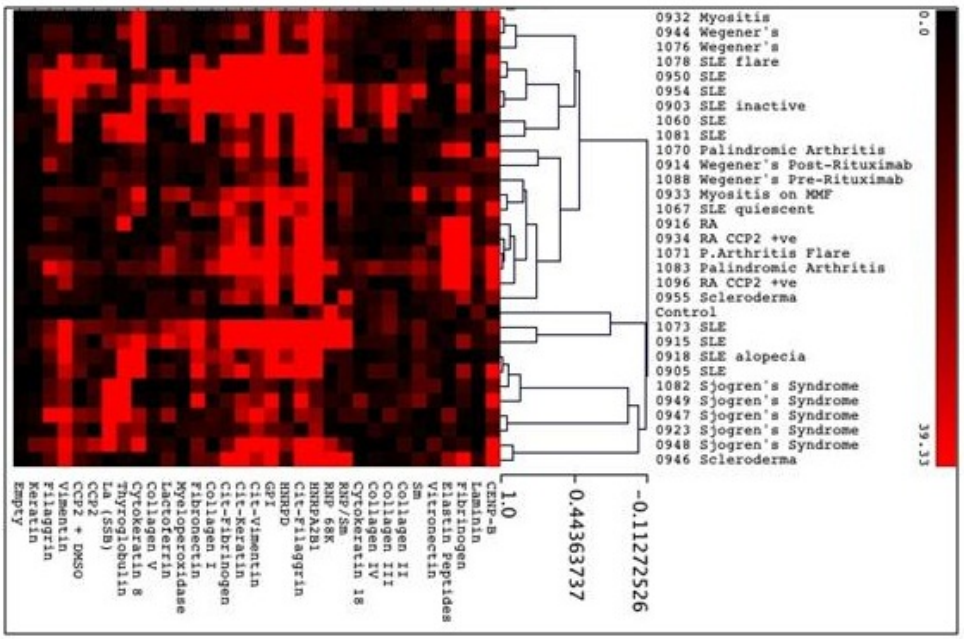

Figure 10

DISCUSSION

Analysis of Sera Tests

Hierarchical Clustering Analysis allows comparisons to be made between related autoimmune diseases (*i.e.*, PA and RA). Further work may aid in understanding similarities between disease aetiologies or what makes them pathologically distinguishable. Incorporating many autoantigens into an assay alongside full utilisation of patient clinical data can lead to novel associations and may identify new disease subgroups (for example, two possible SLE subgroups).

Microarrays also have a potential role in monitoring treatments. Comparative analysis of patients over treatment periods were made and current research indicates there is often a relationship between autoantibody titre and clinical presentation. By monitoring titres before and during treatment, scientists can potentially discover new drug mechanisms and there is a possibility to tailor treatments to an individual's own needs if current regimens are ineffective.

Although evidence associates both RA and COPD with smoking, it would be highly unrealistic to associate RA with COPD in terms of autoantibody targeting from this study; further testing with more samples is needed.

Limitations

Patient samples available to carry out this study were limited. Future research should involve screening with a more even spread of samples on a wider scale. Another limitation was to only use an IgG secondary antibody. Microarrays allow simultaneous detection of multiple antibody isotypes by incorporating multicolour fluorescent detection with anti-isotype specific secondary antibodies[21]. Incorporating this would help identify associations between isotype and pathogenesis, a concept currently under investigation in RA[15,18].

Future Work

- To permit testing for even more autoantibodies, additional antigens can be added to the microarray: many spots remained empty and the design can compress to 20x20 grids. Hueber *et al*. describe an interesting concept, however, that despite best efforts, the number of autoantigens on an array will never be representative of the vast number of expressed proteins in a synovial joint[16].
- Recall antigens that the majority of individuals have immunity towards (*i.e.*, Epstein - Barr virus), can be added to act as positive controls, as is currently undertaken in Paediatric ELISAs.
- It has been argued through three hypotheses ('Citrulline Specific', 'Peptide Specific' and 'Antigen Specific'[11]) that CCPs are not representative of the citrullinated epitopes that exist in vivo. There is, therefore, a growing importance to discover and include the exact citrullinated epitopes acting as ACPA targets to develop more effective and sensitive assays[19].

- Sera samples from healthy individuals would allow a full comparison between disease and non-disease states and give scientists clues as to the role of tolerance towards certain antigens in autoimmune conditions.
- Results should be compared to current ELISA techniques. This would indicate whether identical results can be achieved within a reduced time and costing scale.

Conclusion

Microarrays compress multiple assays into a small space equivalent to a single well of an ELISA, allowing simultaneous testing for autoantibodies to multiple autoantigens in a very confined area. The procedure demonstrates a proof of purpose, with a promising potential to allow accurate diagnosis of RA and other autoimmune conditions. With improvement, there seems to be the capability of diagnosing RA in early stages, complying with NICE guidelines [4,7]. Biomarker profiles can be generated that may relate to symptom severity, treatment response or disease classification and progression[21]. The new method is less time-consuming and less costly, yet maximises patient data collection to unprecedented levels. The minimal volume of blood required also has great significance in terms of seriously ill patients and children. There is a potential to develop the technique further using microfluidics devices, which could take the test into GP clinics. Microarrays could also aid in diagnosis of 'mystery' autoimmune conditions, through elimination of possible candidate diseases or the creation of novel biomarker profiles. It is evidently clear, therefore, that protein microarrays have pivotal future roles in many aspects of immunology.

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