Autoimmunity, autoantigens, autoantibodies
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From Proteomics to Molecular Epidemiology: Relevance of Autoantibodies

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Preface

Autoimmune phenomena are a central pathogenetic principle involved in induction, progression and perpetuation of a broad range of diseases. Systemic autoimmune diseases are chronic, often not curable and have an important impact on the life quality of patients. Despite all progress in immunological research, etiology of most autoimmune diseases still remains unclear. Beside systemic autoimmune diseases, organ-specific and vascular diseases as well as tumors are associated with a variety of immunopathological phenomena. Much has been done in the last years to improve diagnosis and patients' treatment in the field of autoimmune diseases. This book reflects the progress in understanding of associations between diseases and autoantibodies from proteomics to molecular epidemiology. The impact of genomics, ribonomics and proteomics on autoantigen and autoantibody research is presented by new findings based on investigating messenger RNP autoantibodies and applications of mass spectrometry and phage display techniques. Conformational autoepitopes in primary biliary cirrhosis, anti-citrulline antibodies in rheumatoid arthritis, GAD65 epitopes and C-terminal epitopes of ribosomal P-proteins represent some improvement in the repertoire of autoantibodies. Novel autoantibodies against cytoplasmic mRNP, glyceraldehyde-3-phosphatase dehydrogenase, translational regulators, small nucleolar ribonucleoprotein complexes and yet unidentified antibody patterns promise improvement in diagnosis of autoimmune diseases, cancer, hepatitis and other diseases. Despite their correlation with diseases, the induction of autoantibodies remains a field of further research. Animal models, investigation of antibody induction by infectious agents, immunization, antigens with molecular similarity, epitope spreading and apoptotic processes and biological therapeutics such as monoclonal antibodies promise fascinating new insights into pathogenetic processes. Pathogenic effects of autoantibodies are illustrated by pathogenetic investigations on ANCA, catalytic antibodies, autoantibodies against gangliosides, receptors. retinal antigens, type II collagen or epitopes in Goodpasture syndrome. New developments and challenges in the field of diagnostic serology have rapidly become apparent. Beside methodological approaches such as systematic analysis of microbial antigens and infection

induced autoimmunity, novel technical solutions for routine diagnostics such as immunofluorescence with transfected cell substrates, multiplexed bead array tests, "LINE" assays, or autoantigen/peptide arrays improve the possibilities of patients' care and clinical research.

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2.
New diagnostic techniques in autoantigen/ autoantibody analyses

New technologies in the detection of autoantibodies

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Introduction

Autoantibodies to intra-cellular antigens are a diagnostic hallmark of autoimmune diseases that include systemic lupus erythematosus (SLE), systemic sclerosis (SSc), Sjögren's syndrome (SjS), rheumatoid arthritis (RA), polymyositis (PM), organ specific autoimmune conditions, and paraneoplastic syndromes. Historically, autoantibody detection and analysis has relied on a number of different technologies such as particle aggregation, immunodiffusion (ID), indirect immunofluorescence (IIF), complement fixation (CF), hemagglutination (HA), counterimmunoelectrophoresis (CIE), radioimmunoassay (RIA), western and dot immunoblotting (IB), ELISA and functional assays that demonstrate inhibition of the catalytic or other functional activity of the antigen. These conventional technologies have limitations because they tend to be labor-intensive, time consuming, limited in throughput, semi-quantitative, and are not adaptable to leading-edge research.

ID has been used in clinical laboratories for over fifty years because it is inexpensive and the results are specific. However, ID lacks sensitivity and can take up to 48 hours before precipitin lines are interpretable (Figure 1). CF can detect antibodies that are relevant to pathogenesis but it is a laborious technique and less sensitive because not all autoantibodies fix complement. RIA is a sensitive technique but requires specialized equipment and facilities to handle and dispose of radioisotopes. IB is sensitive and allows precise identification of autoantibody targets in crude or purified tissue preparations, but it is costly, time consuming and not all autoantibodies are detected by this technique. IP protocols that use extracts from radiolabeled cells are not suitable

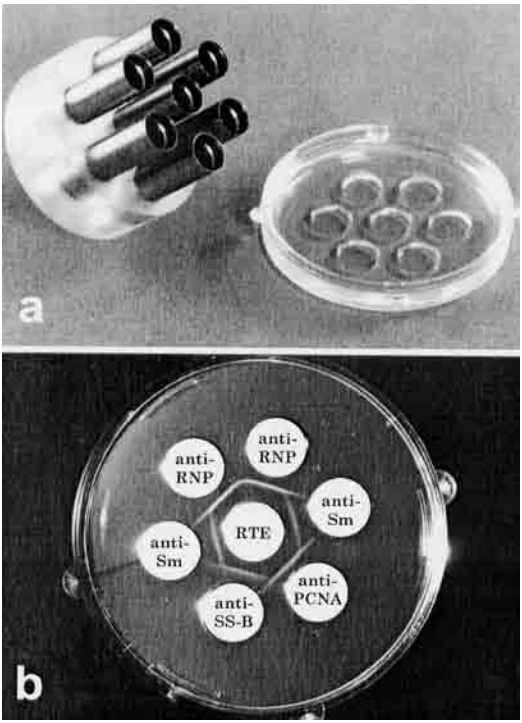

Fig. 1: Tools of the trade for autoantibody testing still in use today date back fifty years. Double (Ouchterlony) immunodiffusion utilized agarose gels and a well cutter (panel a). An antigen source, such as extracts of acetone powdered rabbit thymus (RTE) or other cell extracts, were placed in the center well and in the flanking wells control and text sera were placed. After incubation for 12-48 hours precipitin lines were visible and the identity of the autoantibody based on lines of identity, such as illustrated by the adjacent anti-RNP sera (panel b). By comparison, sera with anti-Sm, -PCNA and –SS-B/La did not produce lines of identity.

for the detection of all autoantibodies, although in some specialized cases IP remains the assay of choice. For example, in the SS-A/Ro system it has been observed that IP techniques are required to identify sera that contain antibodies reacting only with the 52 kDa antigen (1) or the 'native' SS-A/Ro particle (2;3). ELISA techniques have rapidly advanced, but highly specific, sensitive and reliable assays that use highly purified or recombinant proteins are expensive and limited by inter-manufacturer and inter-laboratory variation of

results (4;5). Some laboratories continue to use CIE for the detection of certain autoantibodies such as SS-A/Ro (6;7), although this technique generally favors high titer sera and often cannot discriminate multiple autoantibody responses that are characteristic of systemic rheumatic disease sera.
Despite the advantages of IP, CIE and ELISA, IIF that utilizes HEp-2 and other cell substrates remains the least expensive technique to perform. Autoantibodies are notably heterogeneous with respect to antigen specificity and polyclonality of the B cell response that leads to their formation. These factors, combined with antibody avidity and expression of IgG subclasses, can account for considerable differences in inter-laboratory sensitivity and specificity of test results. The use of IIF to detect antibodies can be limited by a lack of specificity and sensitivity when the antigen in question is of low abundance (8) or there is diffusion of the antigen from the cells during fixation (9). These factors have resulted in significant lot to lot variation from the same manufacturer. Another limitation of current IIF substrates is that there are significant differences in the sensitivity of different commercially available substrate kits to detect different antibodies.
Compared to ELISA and newer technologies, IIF lacks sensitivity and the ability to distinguish autoantibodies to specific molecular targets (10). On the other hand, an advantage of IIF over homogeneous assays such as ELISA, is the ability to simultaneously detect autoantibodies in a single serum. For example, sera from primary biliary cirrhosis typically have anti-mitochondria antibodies but may also contain autoantibodies that react with other relevant antigens such as nuclear pore complexes and centromere proteins (CENPs) that may have additional diagnostic and prognostic importance.
Newer test systems and assays include cells transfected with cDNAs of antigens of interest, "LINE" assays, solid-phase antigen arrays and laser bead arrays where relevant native or recombinant antigens are bound to a solid phase matrix. These new advances are only the beginning of what is becoming a rapid succession of more novel technologies such as microfluidics and nanotechnology. An obvious goal for these technologies is to enhance reliability, sensitivity and cost-effectiveness. Historical analyses suggest that any gains in accuracy obtained with new technologies may be clouded by a need to re-evaluate current paradigms of the diagnostic and prognostic specificity of autoantibodies.

Transfected cell substrates

On most commercial HEp-2 cell preparations, human anti-SS-A/Ro antibodies give a fine speckled nuclear staining pattern (1;9). Although this staining pat-

tern on untransfected cells is quite distinctive, other autoantibodies can produce similar patterns of staining and the presence of other autoantibodies can mask the SS-A/Ro staining pattern. One approach to the problem of sensitivity and specificity of the SS-A/Ro system is the technique of transfection where the appropriate gene(s) (i.e. 60 kD SS-A/Ro gene) are inserted into mammalian cells. This technique introduces multiple copies of the gene, which under the control of specific promoter-regulator genes (11) leads to "over-expression" of the gene of interest. This approach has been utilized in the manufacture of an IIF kit which utilizes the widely used HEp-2 cells transfected with a 60 kD SS-A/Ro cDNA (12). The staining pattern on transfected and overproducing cells is uniquely found in only a portion of the cells in a single high powered field (Figure 2). This feature is helpful because it permits identification of staining by other autoantibodies in the same field of view as the characteristic staining pattern of SS-A/Ro on transfected cells. The variation in 60 kDa SS-A/Ro expression is due to a number of factors including the cell cycle and regulation of expression of the promoter adjacent to the transfected gene (12).

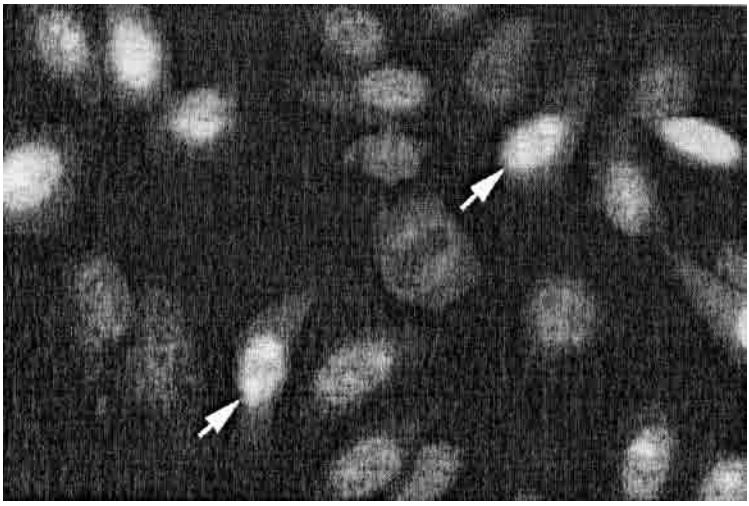

Fig. 2: Indirect immunofluorescence of human sera containing antibodies to the 60 kD SS-A/Ro antigen on HEp-2 cells that were transfected and over-expressing the respective antigen. The identification of a positive reaction is characterized by intense nuclear staining of approximately 25% of the cells (arrows). The staining pattern was typically strongest in nucleus (speckled) and nucleolus, and weaker cytoplasmic staining can also be seen. Original magnification x400.

HEp-2 cells transfected with a 60 kDa human cDNA have been shown to be sensitive and specific substrates for the detection of SS-A/Ro antibodies (13-19). The development of a HEp-2 substrate that detects SS-A/Ro autoantibodies has several advantages. First, a substrate which can be used to screen sera for the presence of a wide variety of nuclear and cytoplasmic antibodies, has the added benefit of being able to detect SS-A/Ro antibodies as well. If the product is competitively priced, the ability to detect SS-A/Ro antibodies with a single test should result in savings for the clinical laboratory and the health care system. Second, because SS-A/Ro antibodies can now be detected by IIF, the turnaround time of reporting could be decreased. Third, the use of the transfected HEp-2 substrate can be adapted to a variety of clinical laboratory settings. For example, laboratories that are not equipped to test samples by IIF can use this substrate in an immunoenzyme assay (20). Fourth, two or three assays are typically required to reliably detect SS-A/Ro antibodies in human sera. The use of this substrate could replace the use of expensive (ELISA, IB) and toxic reagents (radioisotopes) to detect SS-A/Ro antibodies in the clinical laboratory. It has been shown that HEp-2 cells transfected with SS-A/Ro are more sensitive than ELISAs that use recombinant proteins (12).
Transfection techniques can also be used for other autoantibody systems. For example, we have used this technique to transfect HEp-2 cells with the paraneoplastic Hu-D and Yo cDNAs (unpublished) and then used these substrates as alternatives to cryopreserved cerebellum sections for the detection of respective autoantibodies.

Line assays

The ability to produce large amounts of purified and recombinant autoantigens has provided an important advantage to the development of new diagnostic technologies. Included in these newer assays are so-called "LINE assays". LINE assays are based on a solid phase matrix such as a nylon membrane or other protein-binding media on plastic backed sheets. Individual recombinant or highly purified antigens are applied as thin lines, the sheets cut into narrow strips of 0.2 – 0.5 cm, and then each strip is used in an identical manner as conventional IB (Figure 3). A control line is included at the top of each strip to allow for alignment and a template that identifies reactive bands is provided to assist with the visual interpretation of the positive results. In some kits, a "cutoff" strip is also provided that permits distinguishing positive from negative reactivity. Commercial kits based on this technology are marketed by Innogenetics (INNO-LIA ANA™, Ghent, Belgium; in North America, Innogenetics Inc., Norcross, GA, USA); Diagnostic Products Corpo-

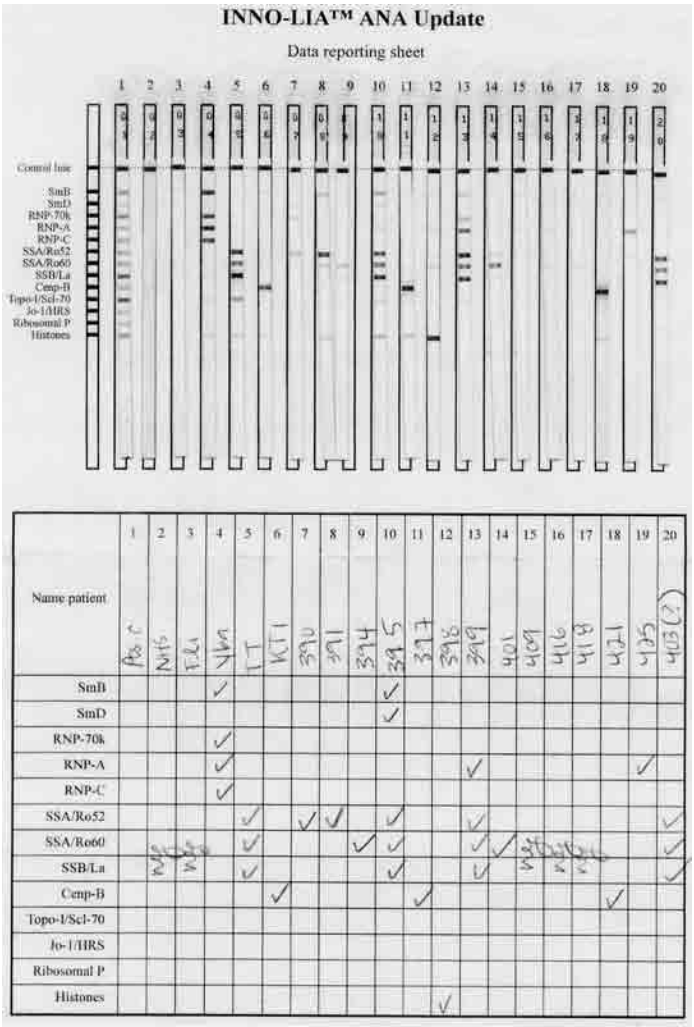

Fig. 3: Analysis of human autoantibodies by IB using a line assay (InnoLIA). A typical analysis of human sera incubated with individual test strips (top panel) is shown. Strips are aligned with the aid of a marker at the top of a strip and reactive antigens on individual bands are identified with the aid of a reference strip (left hand lane). The identification of a reaction as positive or negative is based on the intensity of the band that must be stronger than the 'background" strip (lane 1). Results are indicated on a check-off sheet (bottom panel) and the results can be archived in a standard three ring binder.

ration (ANA LINE Blot, Los Angeles, CA, USA), and by SLR Research (ENA ImmuStrip™, Carlsbad, CA, USA).
Some of these assays have been validated in multicenter evaluations and, for the most part, have been shown to be a reliable alternative to ELISA and western immunoblotting (21). The advantages are a rather impressive in that up to 20 of the more common autoantigens can be assayed in a single test. Unlike conventional immunoblotting where total or soluble cell proteins are blotted onto a membrane, the separation of these antigens into clearly demarcated lines permits rapid identification of those antigens that are reactive with individual sera (Figure 3). The ability to rapidly determine multiple reactivities in a single serum sample has obvious advantages over a homogeneous ELISA or time-consuming immunodiffusion assays (22). Some of the shortcomings of LINE assays are difficulties in adapting the technology to a high volume laboratory, although an automated immunoblotting apparatus is available. In addition, borderline results are often difficult to interpret as positive or negative. Densitometry of the reactive bands might be helpful, but this adds another rather time- and labor-intensive step to the process.
A useful application of LINE assays is to analyze "difficult" sera. For example, from time to time the clinical laboratory encounters sera that appear to have multiple reactivities (i.e. Sm + U1-RNP + SS-A) or the presence of autoantibodies that might otherwise be unanticipated based on publications. These sera are very difficult to analyze using immunodiffusion or counterimmunoelectrophoresis and multiple ELISA assays are required to sort out the autoantibody complexity. In addition, in some instances the autoantibody result may not be in agreement with clear clinical findings (i.e. ANA-negative SLE, SjS, SSc). In this setting LINE assays have provided useful insight into the presence of autoantibodies that might not be anticipated (23) or detected by other assays (13).

Autoantigen arrays

Microchip/membrane spotted arrays

The autoantigen microarray fundamentally uses equipment and software developed for DNA microarrays. In practice, multiple antigens or reactive epitopes are printed onto an array surface in a predetermined configuration. Performing an assay using a patient serum sample or other biological fluids on this substrate allows for the simultaneous and rapid detection of multiple autoantibodies. A recent report of this technology concluded that the use of

autoantigen microarrays represents a powerful tool to study the specificity and pathogenesis of autoantibody responses (24).
Commercialization of this technology to the clinical diagnostic market shows promise. In the future, typical applications will provide an entire system that includes the relevant pre-coupled or spotted arrays, the equipment for sample handling and performance of the test, and software to analyze and report the results. The challenge is to produce consistent, repeatable, reliable and economical tests that rely on manufacturing arrays in a controlled environment. One of the keys to this technology will be the ability to use a single array for numerous tests. This means the stability of antigens after stripping and 're-charging' the array will be important factors.

Laser microbead array

The laser bead microarray platform is a variation of the array concept that has already entered the market. This technology is largely based on the Luminex 100 equipment (Luminex Corp., Austin, TX) that incorporates laser flow technology to analyze reactants in a microtiter format. The key component of this technology is microspheres that are available in up to 100 different laser-reactive colors (Figure 4). In practice, an antigen of interest is chemically coupled to a specific colored microsphere. A second or third antigen is coupled to a microsphere of a different color. After the coupling and stabilizing reactions, the antigen-coupled spheres can be combined into a single microtiter well to provide a single assay that has the ability to detect multiple antibodies in a single specimen. The test sample (serum or other biological fluid) and a fluorochrome-coupled secondary antibody are then added to the well. The sample is then analyzed using principles that are similar to flow cytometry with one laser identifying the specific antigen-coupled bead passing through the path and a second laser determining the presence and quantity of secondary antibody bound to the bead. The data is displayed in a number of formats and can be expressed as a quantitative analysis of multiple analytes and the relative amount of antibody bound to the antigen.
The advantages of this platform include the capacity to analyze a complex array of autoantibodies in a single sample with speed and precision. At least one kit for simultaneously detecting up to six autoantibodies relevant to systemic rheumatic diseases is currently available (ENA Profile 6: Inova Inc., San Diego, CA). Early studies of this kit showed a high level (>90%) of agreement with conventional techniques (unpublished data). Advantages of this technology includes small amounts (5 µl) of serum required for the assay and test sera and secondary antibody are added as a single step with no washing of beads

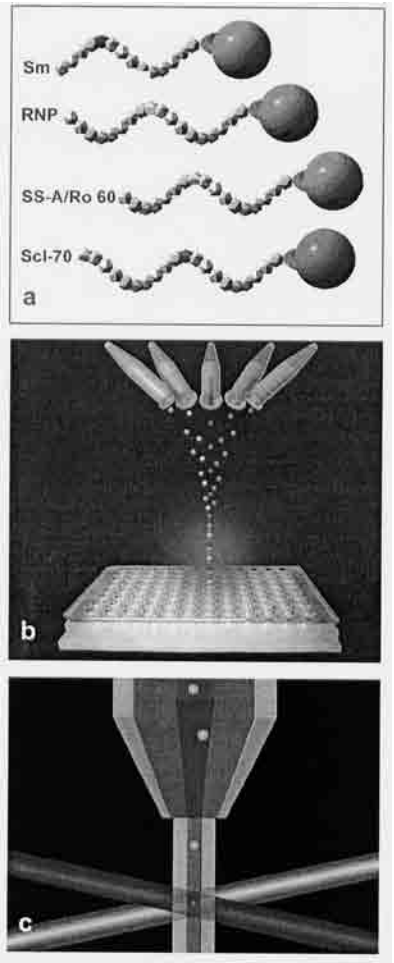

Fig. 4: Laser bead antigen arrays are developed by chemically coupling the antigen of interest onto microbeads that have incorporated a laser reactive dye (panel a). In practice each antigen is coupled to a differently colored bead and the antigens on different colored beads can then be combined into a single microtiter well (panel b). The test serum and secondary antibody coupled to a fluorochrome are then added to the well and analysis is conducted with technology that is similar to flow cytometry. One laser determines the identity of the bead (color = antigen of interest) and another laser simultaneously scans the bead to determine the presence and amount of fluorochrome-labeled secondary antibody (panel c).

required. These factors combine to generate rapid test results since analysis of multiple antibodies in a single well typically takes less than two minutes. In addition, test reports can be automatically generated through Excel or other database management software.

Microfluidics

Microfluidics technology, also referred to as "lab on a chip", is currently under development and evaluation (25;26) (27;28). This technology is adaptable to a number of different configurations and it involves the miniaturization and integration of liquid and biological fluid handling and analysis on microfabricated devices (29-31). These devices are manufactured by etching or molding interconnecting channels onto glass, quartz, silicon or other solid phase matrices. The equipment and software is designed to provide a hard-wired testing protocol. The design can include pumps, valves, extraction and separation modules, reaction chambers, and channels that permit rapid processing of test samples (Figure 5). Unlike conventional laboratory techniques that require human intervention at multiple steps to manipulate of evaluate samples, this technology has the potential to be virtually hands-free.

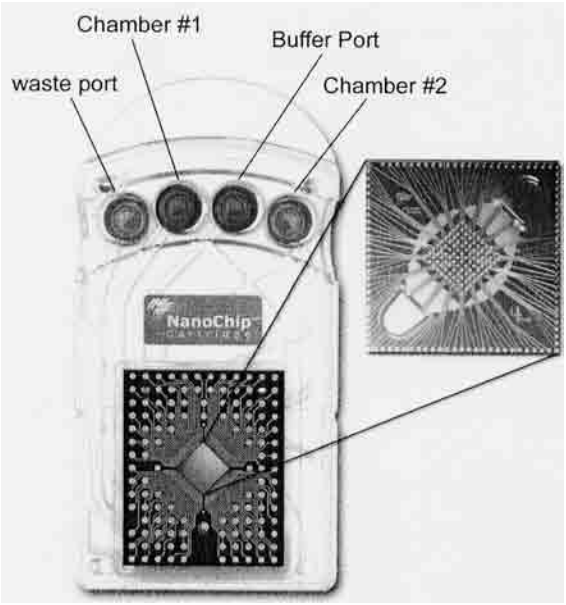

Fig. 5: A typical microfluidics, microarray, nanochip cartridge (permission to copy NanoChip™ Cartridge, Nanogen, Inc., San Diego CA). These devices can be adapted to conduct antigen-antibody analyses by placing antibody or serum in chamber #1 or in chamber #2. The microarray would include microspots of relevant antigens.

In principle, antigen, antibodies, buffers and analytes are moved, switched and combined under the influence of electrokinetic forces so that unique physicochemical properties can be detected when two proteins form an antigen-antibody complex. Future microfluidics platforms may include a module that will physically prevent blood cells and other particulate debris from entering the reaction chamber (32). In this way a small amount of whole blood (i.e. finger prick) can be utilized without the need for to process blood samples to obtain serum.
The advantage of microfluidics includes the combined potential of a large number of parallel channels, short distances, and high electrical fields that will increase throughput by several orders of magnitude. The integration of multiple analytes on a single chip and the relatively small amount of reagents and materials required for a single analysis holds promise for a very rapid and relatively inexpensive technology to detect autoantibodies.
One application of microfluidics is based on capillary electrophoresis (26;28). This technology will allow antigens and antibodies to be in an aqueous environment thus preserving protein structure and theoretically allowing for optimal antigen-antibody interactions *in vitro*. This platform is similar to other microfluidics platforms wherein the antigen and antibody 'streams' are brought together and mixed in a common microchannel and allowed to react for various times. The antigen-antibody conjugates are then separated or distinguished from unreacted proteins and detected on the basis of flow characteristics that are related to electrophoresis and electroosmosis when an external electric field is applied. This technology requires very small volumes (i.e. picoliters) and quantities (i.e. nanograms) of reactants for each assay (28). This is in contrast to the solid phase detection microarray systems where nanoliter spot sizes require high sample density. This platform has the flexibility to combine an assay with simple antigen-antibody binding event detection and measurement of antibody binding affinity and avidity. Perhaps most important to the clinical laboratory, the detection time for this technology is extremely rapid. For example, in a single assay the analysis is limited only by the transport of samples from the reservoirs, the maximum electric field that can be used, and the rate of reaction of antibody with antigen. In practice, this can be achieved in approximately 500 milliseconds.

Nanotechnology and nanoengineering

Nanotechnology is defined as the creation of functional materials, devices, and systems through the control of matter as a scale of 1 – 100 nanometers. In addition, this technology is able to exploit the novel properties of and phe-

nomena of antigen-antibody binding at the atomic level. This technology not only provides the potential of the ultimate in microfluidics diagnostics *ex vivo* but it also provides the opportunities to develop diagnostic tools that can be targeted to specific organs and to specific cells within organs. Clearly this is a major focus for the future of diagnostics and therapeutics alike.

Summary

Advances in technologies are rapidly changing the approach to diagnostic serology. The integration and adoption of these new technologies has promise of improving the sensitivity, specificity, speed and cost of autoantibody testing. However, these technologies will also produce new and complex data sets that may require revision of existing diagnostic and therapeutic paradigms. The widespread use of newer technologies requires dialogue (33) and the input of clinicians, engineers, basic scientists and the standardization of the assays based on highly characterized and standardized sera and reagents.

Abbreviations

ANA, antinuclear antibody; CIE, counterimmunoelectrophoresis; CENP, centromere protein; CF, complement fixation; ELISA, enzyme linked immunoassay; ENA, extractable nuclear antigens; HA, hemagglutination, IB, immunoblotting; ID, immunodiffusion; IP, immunoprecipitation; kDa, kiloDalton; PM, polymyositis; RA, rheumatoid arthritis, RIA, radioimmunoassay; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; SS-A/Ro, Sjögren's syndrome antigen A; SSc, systemic sclerosis.

References

1. Chan EKL, Buyon JP. The SS-A/Ro antigen. In: Van Venrooij W, Maini RN (Eds.) Manual of Biological Markers of Disease (pp. 1-18). Dordecht, The Netherlands: Kluwer Academic Publishers, 1994
2. Boire G, Craft J. Human Ro ribonucleoprotein particles: characterization of native structure and stable association with the La polypeptide. J Clin Invest 1990; 85: 1182-1190
3. Boire G, Lopez-Longo F-J, Lapointe S, Menard H-A. Sera from patients with autoimmune disease recognize conformational determinants on the 60-kd Ro/SS-A protein. Arthritis Rheum 1991; 34: 722-729

4. Tan EM, Smolen J, McDougal JS, Butcher BT, Conn D, Dawkins R et al. A critical evaluation of enzyme immunoassays for the detection of antinuclear antibodies of defined specificities. I. Precision, sensitivity and specificity. Arthritis Rheum 1999; 42: 455-464
5. Tan EM, Smolen JS, McDougal JS, Klippel JH, Fritzler MJ, Gordon TP et al. A critical evaluation of enzyme immunoassay kits for the detection of antinuclear antibodies of defined specificities. II. Potential for quantitation of antibody content. J Rheumatol 2002; 29: 68-74
6. Manoussakis MN, Kistis KG, Liu X, Aidinis V, Guialis A, Moutsopoulos HM. Detection of anti-Ro(SS-A) antibodies in autoimmune diseases: comparison of five methods. Br J Rheumatol 1993; 32: 449-455
7. Meilof JF, Bantjes I, de Jong J, Van Dam AP, Smeenk RJT. The detection of anti-Ro(SS-A) and anti-La(SS-B) antibodies. A comparison of counterimmunoelectrophoresis with immunoblot, ELISA, and RNA-precipitation assays. J Immunol Meth 1990; 133: 215-226
8. Hendrick JP, Wolin SL, Rinke J, Lerner MR, Steitz JA. Ro small cytoplasmic ribonucleoproteins are a subclass of La ribonucleoproteins: further characterization of the Ro and La small ribonucleoproteins from uninfected mammalian cells. Mol Cell Biol 1981; 1: 1138-1148
9. Harmon CE, Deng JS, Peebles CL, Tan EM. The importance of tissue substrate in the SS-ARo antigen-antibody system. Arthritis Rheum 1984; 27: 166-173
10. Fritzler MJ. Autoantibody testing: Procedures and significance in systemic rheumatic diseases. Meth Achiev Exp Pathol 1986; 12: 224-260
11. Thomsen DR, Stenberg RM, Goins WF, Stinski MF. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc Natl Acad Sci USA 1984; 81: 659-663
12. Keech CL, McCluskey J, Gordon TP. Transfection and overexpression of the human 60-kDa Ro/SS-A autoantigen in HEp-2 cells. Clin Immunol Immunopath 1994; 73: 146-151
13. Fritzler MJ, Hanson C, Miller J, Eystathioy T. Specificity of autoantibodies to SS-A/Ro on a transfected and overexpressed human 60 kDa Ro autoantigen substrate. J Clin Lab Anal 2002; 16: 103-108
14. Baier JE, Poehlau D. Is α-methyldopa-type autoimmune hemolytic anemia mediated by interferon-gamma. Ann Hematol 1994; 69: 249-251
15. Keech CL, Howarth S, Coates T, Rischmueller M, McCluskey J, Gordon TP. Rapid and sensitive detection of anti-Ro (SS-A) antibodies by indirect immunofluorescence of 60 kDa Ro HEp-2 transfectants. Pathology 1996; 28: 54-57
16. Pollock W, Toh BH. Routine immunofluorescence detection of Ro/SS-A autoantibody using HEp-2 cells transfected with human 60 kDa Ro/SS-A. J Clin Pathol 1999; 52: 684-687
17. Bossuyt X, Meurs L, Mewis A, Marien G, Blanckaert N. Screening for autoantibodies to SS-A/Ro by indirect immunofluorescence using HEp-2000TM cells. Ann Clin Biochem 2000; 37: 216-219

18. Morozzi G, Bellisai F, Simpatico A, Pucci G, Bacarelli MR, Campanella V et al. Comparison of differrent methods for the detection of anti-Ro/SSA antibodies in connective tissue diseases. Clin Exp Rheumatol 2000; 18: 729-731
19. Peene I, Van Ael W, Vandenbossche M, Vervaet T, Veys E, De Keyser F. Sensitivity of the HEp-2000 substrate for the detection of anti-SSA/Ro60 antibodies. Clin Rheumatol 2000; 19: 291-295
20. Fritzler MJ, Wall W, Gohill J, Kinsella TD, Humbel R-L. The detection of autoantibodies on HEp-2 cells using an indirect immunoperoxidase kit (Colorzyme). di 1986; 4: 217-221
21. Meheus L, Van Venrooij WJ, Wiik A, Charles PJ, Tzioufas AG, Meyer O et al. Multicenter validation of recombinant, natural and synthetic antigens used in a single multiparameter assay for the detection of specific anti-nuclear antibodies in connective tissue disorders. Clin Exp Rheumatol 1999; 17: 205-214
22. van der Linden MW, Rudi GJ, Westendorp MZ, Meheus L, Huizinga TWJ. Autoantibodies within families of patients with systemic lupus erythematosus are not directed against the same nuclear antigens. J Rheumatol 2001; 28: 284-287
23. Rutjes SA, Vree Egberts WT, Jongen PH, Pruijn G, Van Venrooij JV. Anti-Ro52 antibodies frequently co-occur with anti-Jo-1 antibodies in sera from patients with idiopathic inflammatory myopathy. Clin Exp Immunol 1997; 109: 32-40
24. Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M, Dean EJ et al. Autoantigen microarrays for multiplex characterization of autoantibody responses. Nature Med 2002; 8: 295-301
25. Robinson WH, Steinman L, Utz PJ. Proteomics technologies for the study of autoimmune disease. Arthritis Rheum 2002; 46: 885-893
26. Sanders GHW, Manz A. Chip-based microsystems for genomic and proteomic analysis. Trends Analyt Chem 2000; 19: 364-378
27. Kniss R. Revolution of the drug discovery process using laboratory-on-a-chip technology. Amer Lab 1998; 30: 40-42
28. Utz PJ. Microfluidics and proteomics. http://www.stanford.edu/group/utzlab/proteomics.htm , 2002
29. Polson NA, Hayes MA. Microfluidics: controlling fluids in small places. Anal Chem 2001; 73 (11): 312A -319A
30. Mitchell P. Microfluidics--downsizing large-scale biology. Nat Biotechnol 2001;19 (8): 717 –21
31. Davies MJ. Microfluidics venture. Trends Biotechnol 2001; 19 (9): 331-332
32. Nicholson P. Big things come in small packages. Biotechnol Focus 1999: 16-29
33. Utz PJ. 21st European workshop for rheumatology research, Vienna, Austria, 1-4 March 2001. Arthritis Res 2001; 3: 237-240

Systematic analyses of microbial antigens and infection induced autoimmunity

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A number of viral and bacterial infections are associated with antibody-mediated autoimmune diseases. Using the SEREX approach (SErological identification of Recombinantly EXpressed antigens), we systematically dissected the autoantibody repertoire of mice after infection with cytopathic or non-cytopathic viruses. cDNA expression libraries prepared from various organs 48-72h after infection were immunoscreened using sera harvested from virus-infected mice. Screening of more than 2,5 x10^6 clones demonstrated that virus-induced autoimmune B cell responses are of broad specificity; e.g., antibody responses against more than 60 autoantigens were detected after Vaccinia virus (VV) infection. Interestingly, the vast majority of the identified autoantigens after VV infection, which include intracellular enzymes, transcription factors, cytoskeletal proteins and surface molecules, have been previously described as autoantigens in humans. Antibody titers against a number of autoantigens were comparable to anti-VV antibody responses. Comparison of serum reactivity against a selected panel of autoantigens after infection with VV, lymphocytic choriomeningitis virus or vesicular stomatitis virus showed that the different virus infections leave distinct pattern of autoreactivity in the infected host. These data indicate that virus infections are responsible for a significant fraction of the autoantibody reportoire and that individual viruses may trigger specific “autoantibody fingerprints”.

Line-assay with recombinant antigens for diagnosis of systemic autoimmune rheumatic diseases

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The goal of this project was to develop an easy-to-use test system with high sensitivity and specificity for detecting circulating autoantibodies in sera of patients with systemic autoimmune rheumatic diseases, like SLE, Sjögren's syndrome, scleroderma, myositis and others. Therefore we have cloned the cDNA of 13 antigens including RNP-70kD, RNP-A, RNP-C, SmB, SmD, SSA-60, SSA-52, SSB, ribosomal P0-protein, PCNA, CENP-B, DNA topoisomerase I (Scl70), and histidyl-tRNA synthetase (Jo1). The cDNAs were expressed heterologously in E. coli bacteria and SF21 insect cells, respectively, and were purified by successive chromatographic procedures. The recombinant proteins as well as native dsDNA and histones were brought on nitrocellulose membrane strips by dot-blot technique. Using this test format we analyzed 380 sera of patients with different types of connective tissue diseases, 50 sera of vasculitis patients, 20 sera of patients with rheumatoid arthritis, 50 with reactive arthritis, and 220 sera of normal subjects. We have found high agreement of our results with those obtained from other available test systems. In addition, this test format serves as a fast method to distinguish between autoimmune and other rheumatic diseases with similar symptoms. The high sensitivity and specificity of this assay and its easy interpretation as well as its simple laboratory procedure makes this assay to a promising tool in the differential diagnosis of autoimmune rheumatic diseases.

Three multiplexed tests for extractable nuclear antigens using the Luminex 100

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We developed convenient multiplexed immunoassays for the detection of autoantibodies to the extractable nuclear antigens Sm, RNP, SSA, SSB, Scl-70 and Jo-1. The results obtained with this new technology were compared to clinical diagnosis and the results found with standard ELISA methodology.

A no-wash multiplexed assay that detects autoantibodies to up to 6 antigens in a single well was developed. Each of the 6 antigens is coated on a unique bead. The appropriate beads are then mixed in a single well as either a 4-plex comprised of Sm, RNP, SSA and SSB, a 5-plex comprised of those 4 antigens plus Scl-70, or a 6-plex comprised of those 5 antigens plus Jo-1. These are called QUANTA Plex tests. Sera from normal blood donors, from patients with clinically defined rheumatic diseases, and from samples with high titers of specific antibodies were tested on all multiplex tests and the corresponding ELISAs.

Samples from 200 normal blood donors were tested, and for every antigen on both technologies at least 99% were negative. The cutoff between negative and positive on the QUANTA Plex tests ranged between 4 and 13 standard deviations above the average of this group. Over 250 samples from patients with systemic lupus erythematosus, scleroderma and Sjogren's syndrome were tested on the QUANTA Plex tests and ELISAs. Compared to ELISA, the relative sensitivity for each QUANTA Plex test was about 95%, and the relative specificity was 98% or higher. Thus, it is not surprising that the ELISA and QUANTA Plex tests had the same clinical sensitivity and specificity. Control sera with antibodies to known infectious disease organisms or irrelevant autoantigens showed the same sensitivity and specificity on the QUANTA

Plex tests and ELISAs. Adding beads with Scl-70 and Jo-1 antigens to the QUANTA Plex ENA Profile 4 did not change the reactivity of any sera to the original 4 antigens.

Conclusion: The 3 QUANTA Plex ENA Profile tests using the Luminex flow cytometer yield sensitivity and specificity nearly identical to ELISA. There are no measurable interactions among the different beads in the same well. Performing all assays for a given sera in only 1 well saves space and time.

The BIOONE® system: The new dosage of autoantibodies

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The concentration of autoantibody in serum from autoimmune patients are determined with several methods existing and available in the market (ELISA with direct coating of autoantigen, Immunoblot, RIA, IFA). We have recently developed a new method, called BIOONE®, using one and only one microtiterplate coated with streptavidin for major autoimmune parameters.
The specific autoantibodies in diluted serum react with soluble biotinylated autoantigen during the first incubation time (30 min. at 37°C). In the same time, the complex formed react directly with the solid phase. After washing, a peroxidase conjugated antibody is added to the wells and incubated (60 min. at 37°C). A last washing step remove the unbound conjugate and specific autoantibodies are traced by incubation with substrate solution (15 min. at 37°C).
We evaluated the BIOONE® system for the diagnosis of AITD (autoimmune thyroid diseases) and ANCA (MPOAb, PR3Ab and mix). Intra-assay precision were found to be < 10% and inter-assay to be < 15%. The clinical results have been also compared with other commercial ELISA and/or IFA technics.

General conclusion, the BIOONE system:

- has a good correlation with the reference methods.
- individualizes the dosage. The autoantigen can be distributed anywhere into the microtiterplate; open to evolution of autoimmune diseases diagnostic (many tests with few patients).

- permits the analysis of aspecific binding with the solid phase (omition of autoantigen during the first incubation time). Aspecific binding against plastic of microtiterplate or streptavidin.
- keeps the native form of autoantigen. No modification of the 3D structure following direct adsorption.
- permits a complete access of autoantigen for autoantibodies.
- optimizes the concentration of autoantigen.

Automatized classification of HEp-2-cell based indirect immunofluorescence patterns by a novel computerized system

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In diagnosis of systemic autoimmune diseases, indirect immunofluorescence with HEp-2-cells presents the major screening method. This procedure allows to scan a broad range of autoantibodies and to classify them by distinct fluorescence patterns. The identification of the patterns is currently done manually by inspecting the slides with the help of a microscope. The major disadvantages of this method are the need for highly specialized technicians and a lack of automatized solutions. Furthermore, need for standardization and documentation is hampered by the common microscopic technique.

We have established a novel system for automatized classification of HEp-2 fluorescent patterns. Designed as an assisting system, representive patterns are acquired by an operator with a digital microscope camera and transferred to a personal computer. A software package was developed based on a novel image analysis and feature extraction algorithm. The generated data set was then subject to machine learning techniques to find out relevant features for a correct classification of autoantibodies. The learning algorithm selected from the data set the most promising features and constructed the structure of a classifier during the learning phase.

Our results show, that identification of positive fluorescence and discrimination between most important autoantibody entities can be performed by this system. The decision tree as generated in the learning phase can be integrated in the laboratory system. To enable the usage of such a system in routine diagnostics, accuracy of this system must be further improved. At the moment, more then 80 percent of samples can be classified in a correct way. Furthermore, overall speed of the system must be enhanced.

We conclude that the established system can be used as an automatic inspection module. The integrated software for machine learning allows to permanently improve reliability of classification as well as number of recognized autoantibody entities.

Simple and sensitive detection of serum autoantibodies to full-length bullous pemphigoid antigen 180[1]

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Introduction

Bullous pemphigoid (BP), the most common autoimmune bullous skin disorder (1,2), is characterized by tissue-bound and circulating autoantibodies to the hemidesmosomal proteins BP180 and BP230 (3,4). Whereas BP230 is localized intracellularly to the hemidesmosomal plaque, BP180 is a type II transmembrane glycoprotein with a C-terminal ectodomain that contains 15 interrupted collagenous domains and spans the lamina lucida of the dermal-epidermal junction (DEJ; reviewed in 5). BP180 is essential for the integrity of the DEJ since mutations in the BP180 gene result in an inherited blistering skin disorder that was characterized by loss of adhesion within the DEJ (reviewed in 6).

The majority of BP sera reveals reactivity with the 16th non-collagenous (NC) domain directly adjacent to the transmembrane portion (NC16A; 7-9). Reactivity of BP sera with other intra- and extracellular sites located on BP180 has also been reported (10-12). The pathogenic relevance of anti-BP180 antibodies has previously been demonstrated by the passive transfer of rabbit anti-BP180 antibodies into neonatal mice (13). In addition, in BP patients, levels of

[1] Presented in detail in Ref. 23.

circulating antibodies to BP180 NC16A have been shown to parallel disease activity (14).
BP is usually diagnosed by the use of several assays, including (1) direct immunofluorescence (IF) microscopy of a perilesional skin biopsy, (2) histopathology of a lesional biopsy, and (3) indirect IF microscopy of patient sera on 1M NaCl-split human skin, i.e. an assay which can distinguish between epidermal and dermal binding of autoantibodies (15; reviewed in 16). These assays, however, have several drawbacks: Both IF microscopy and histopathology require skin biopsies and indirect IF on salt-split skin does not distinguish between different specificities of autoantibodies directed to different components of the DEJ and strongly depends on the quality of human split-skin that may vary considerably. More recently, recombinant fragments of BP180 were employed for the detection of serum autoantibodies in BP by Western blotting (WB) and enzyme linked immunosorbent assay (ELISA; 7-12,17-19). The disadvantage of these assays is that recombinant fragments were frequently generated in prokaryotic cells, thus conformation-dependent epitopes may not be expressed. In addition, they do not encompass the full-length (FL) BP180 molecule.
In the present study, we expressed FL-BP180 as a type II transmembrane protein by the use of an eukaryotic expression system and, subsequently, developed a simple and highly sensitive and specific assay for the detection of circulating antibodies to BP180 that may be used in any routine IF laboratory.

Results

Full-Length (FL) BP180 was expressed as Type II Transmembrane Protein in Sf21 Insect Cells. Sf21 insect cells were infected by recombinant baculovirus encoding FL-BP180 (MOI = 4.0; 20), fixed with acetone, and labeled with various mouse mAb to BP180 (see schematic diagram in Fig. 1; 21, 22). By confocal laser scanning microscopy FL-BP180 was shown to be expressed uniformly within the cell membrane of Sf21 insect cells (data not shown; 23). In order to demonstrate that FL-BP180 is expressed as type II transmembrane protein (like in the human keratinocyte), FL-BP180 expressing Sf21 insect cells and cells infected with a negative control construct were incubated with mAbs to BP180, respectively. Analysis by flow cytometry revealed that mAbs against the extracellular domain of BP180 were bound, but not the mAb against the intracellular domain unless the cell membrane of the FL-BP180-expressing insect cells had been permeabilized prior to incubation with mAb directed to the intracellular domain (data not shown; 23).

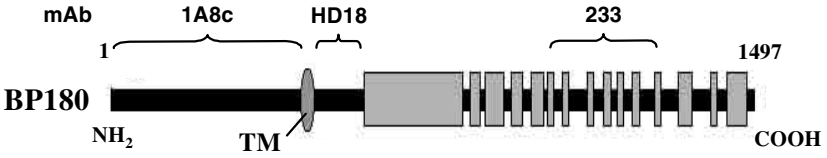

Fig. 1: Schematic diagram of recombinant and cell-derived forms of human BP180. The N-terminal cytoplasmatic domain of BP180 (left side) and the C-terminal ectodomain (right side) consisting of 15 interrupted collagen domains (gray boxes) are shown. Amino acid numbers are depicted next to the proteins. The epitopes recognized by the mouse monoclonal antibodies (mAb) to human BP180 used in this study are indicated at the top of the panel. TM, transmembrane portion of BP180.

Detection limits of the Sf21-based immunofluorescence assay

In order to quantitatively determine the sensitivity of the IF assay using FL-BP180-expressing Sf21 insect cells, binding of the mouse mAbs 233, HD18, and 1A8c was studied at serial dilutions of 1:5 to 1:20,480 by indirect IF microscopy (23). While no staining was seen with the control cells infected with the negative control construct (encoding the hexa-histidine tag; data not shown), the mAbs labeled the FL-BP180-expressing Sf21 insect cells (Fig. 2). The sensitivity of the Sf21-based IF assay was compared with (1) indirect IF microscopy on salt-split skin, (2) WB of FL-BP180 generated in Sf21 insect cells, and (3) WB of recombinant BP180 NC16A (Table 1; 23). It is evident that the eukaryotic IF assay was more sensitive than the conventional assays. Based on the IgG concentration in mAb preparations and the data in Table 1, the detection limit of the eukaryotic IF assay was calculated to be 72 ng/ml (mAb233), 17 ng/ml (mAbHD18), and 16 ng/ml (mAb1A8c).

Detection of serum antibodies to BP180 in patients with bullous pemphigoid

To further characterize the sensitivity and specificity of the Sf21-based IF assay 65 sera of BP patients with circulating antibodies to the DEJ as detected by indirect IF microscopy on salt-split human skin were examined. As shown by screening experiments, nonspecific staining occurred in about a third of human sera derived from both patients and healthy controls. Nonspecific IF

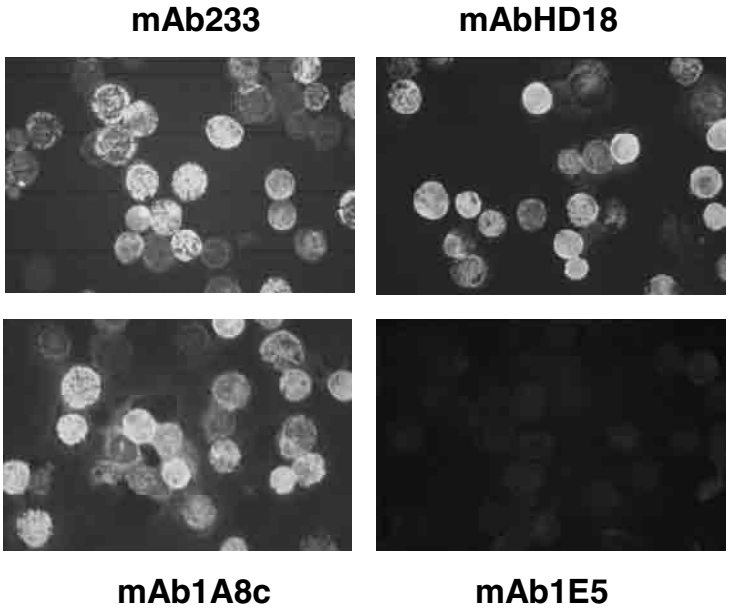

Fig. 2: Full-length BP180 expressed in Sf21 insect cells is recognized by mouse monoclonal antibodies directed against both intra- and extracellular epitopes on human BP180. When Sf21 insect cells were infected with baculovirus encoding full-length BP180 at a multiplicity of infection of 4.0, about 30% cells were strongly stained after fixation with acetone and incubation with various mouse monoclonal antibodies (mAb) to human BP180 (for details see Fig. 1). Non- or weakly-stained cells served as internal negative control. No staining was seen with mAb1E5 directed against human BP230.

reactivity varied considerably between the different sera and was present in both FL-BP180-expressing Sf21 insect cells and cells infected with the control construct. However, when the sera were pre-incubated with non-infected Sf21 insect cells, the unspecific background staining could greatly be reduced (Fig. 3; 23). After pre-adsorption 58 of 65 BP sera (89%) were reactive. In contrast, no reactivity was observed with sera from patients with pemphigus vulgaris (n = 15), pemphigus foliaceus (n = 5), epidermolysis bullosa acquisita (n = 5), atopic dermatitis (n = 7), contact dermatitis (n = 3), systemic lupus erythematosus (n = 5) as well as with normal human sera (n = 30). Results of BP sera obtained by the Sf21-based IF assay were then compared with WB of (1) FL-BP180, (2) a 55 kD C-terminal fragment of BP230, and (3) the cell-derived

Tab. 1: Sensitivity of immunofluorescence (IF) microscopy of full-length (FL)-BP180-expressing Sf21 insect cells compared with the sensitivity of current assays using serial dilutions of mouse monoclonal antibodies directed to various intra- and extracellular epitopes on BP180

Designation	Immunofluorescence		Immunoblotting	
	FL-BP180[1]	Salt-split Skin	FL-BP180	BP180 NC16A
233	1,280	1,280	20	na
HD18	640	20	5	640
1A8c	2,560	640	10,240	na

[1]expressed in Sf21 insect cells fixed with acetone; na, not applicable; NC16A, extracellular portion of the 16th non-collagenous domain of BP180

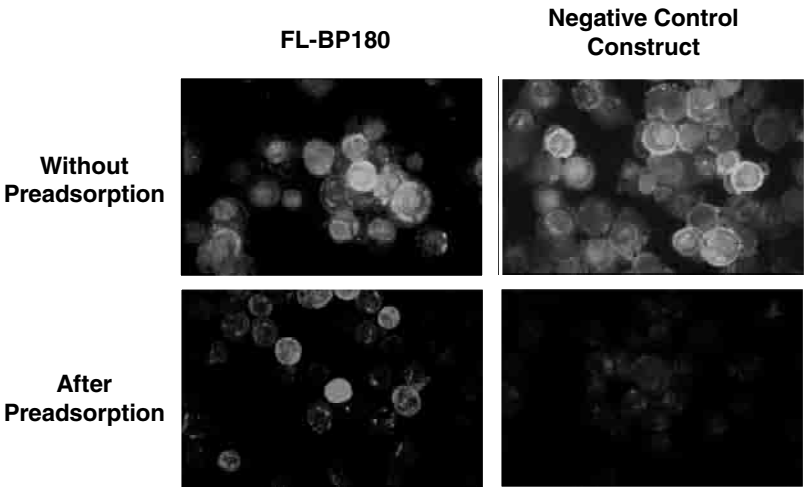

Fig. 3: Preadsorption of BP sera with non-infected Sf21 insect cells reduced unspecific background immunofluorescence (IF) staining of negative control construct-infected Sf21 insect cells. Sf21 insect cells were infected with baculovirus encoding full-length BP180 (FL-BP180) or a hexa-histidine peptide (negative control construct). Without preadsorption, the bullous pemphigoid (BP) serum strongly labeled FL-BP180-expressing Sf21 insect cells. However, unspecific staining is seen in cells infected with the negative control construct. After preadsorption of the BP serum with -non-infected Sf21 insect cells, the serum still labeled FL-BP180-expressing insect cells but nonspecific staining of negative control construct-infected cells was greatly reduced.

Tab. 2: Detection of antibodies to BP180 in 65 bullous pemphigoid sera that contained antibodies against components of the dermal-epidermal junction determined by the eukaryotic immunofluorescence assay and conventional assays

FL-BP180 Sf21 Insect Cells[2]	Immunoblot	NC16A[1]	LAD-1[1]	BP230 55 kD[1]
58/65	39/65	49/65	38/65	38/65
89%	60%	75%	58%	58%

[1]analyzed by immunoblotting; [2]detected by indirect immunofluorescence microscopy; FL-BP180, full-length BP180; NC16A, extracellular portion of the 16th non-collagenous domain of BP180; LAD-1, linear IgA disease antigen (cell-derived ectodomain of BP180); BP230-55 kD, 479 amino acid C-terminal stretch of BP230

soluble ectodomain of BP180 (LAD-1; Table 2; 23). Comparison of the data shows that indirect IF microscopy of FL-BP180-expressing Sf21 insect cells appears to be the most sensitive tool for the detection of circulating antibodies against BP180.

Reactivity of stored FL-BP180-expressing Sf21 insect cells

FL-BP180-expressing Sf21 insect cells and cells infected with the negative control construct were fixed with acetone and stored at 4-8°C or -20°C for 3 months. After thawing, cells were labeled with mouse mAbs 233, HD18, and 1A8c to BP180 (for details see Fig. 1) at dilutions of 1:320 and 1:640 (23). Reactivity was compared with cells that were prepared on the same day and fixed with acetone and scored from "+++" for bright staining to "+/-" for faint staining (Table 3; 23). When cells were stored at 4-8°C, no or only weak staining was observed; in contrast, reactivity to BP180 was conserved in FL-BP180-expressing cells when stored at -20°C. However, the reactivity was about one titre step lower compared with freshly prepared cells (Table 3).

Discussion

The data presented here show that the indirect IF microscopy of full-length BP180-expressing Sf21 insect cells is more sensitive for anti-BP180 antibodies than conventional assays, such as indirect IF microscopy on human salt-split

Tab. 3: BP180 expression is largely conserved upon storage of full-length BP180-expressing Sf21 insect cells at -20°C for 3 months

Designation[1]	Fresh[2]	-20°C[3]	4-8°C[3]
mAb233	+++[4]	++	+/-
mAbHD18	+	+/-	-
mAb1A8c	+++	++	-

[1]mouse monoclonal antibodies (mAb) 233, HD18, and 1A8c directed against various epitopes on human BP180 (for details see Fig. 1) were tested at dilutions of 1:320 and 1:640; [2]prepared, fixed with acetone, and stained on the same day; [3]stored for 3 months after fixation with acetone; [4]immunofluorescence reactivity was scored from "+++" for bright to "+/-" for faint staining. When no staining was seen, the score was "-".

skin, Western blotting of recombinant and cell-derived fragments of BP180, as well as Western blotting of FL-BP180 produced in Sf21 insect cells. This conclusion can be drawn from the low detection limit of the eukaryotic IF assay as determined by serial dilutions of BP180-specific mouse monoclonal antibodies and from the reactivity with a large panel of BP and negative control sera. About 90% of BP sera were found to be positive when unspecific background reactivity had been removed by pre-incubation of the sera with non-infected Sf21 insect cells. Background reactivity, detected in about one third of human sera (both patients and healthy volunteers), could be greatly reduced by (1) using a MOI of 4.0, (2) preadsorption of sera with non-infected Sf21 insect cells, and (3) controlling the preadsorption procedure by incubation of sera with control construct-infected Sf21 insect cells.

In contrast to the Sf21-based IF assay, in the case of Western blotting of recombinant BP180 NC16A and keratinocyte-derived LAD-1, only 75% and 60% of BP sera, respectively, were reactive. Similar reactivity for BP180 NC16A and LAD-1 in BP sera was reported by other authors (7, 9, 18, 19, 20, 24). Hata et al., using the recombinant ectodomain of BP180, found reactivity in about 85% of BP sera (25). With the same technique, 92% of BP sera were reported positive by Haase et al. (26). However, the protein was extracted under strongly denaturing conditions; thus it is unlikely that the physiological conformation of human BP180 was maintained. In addition, only sera of patients with generalized BP were tested (26). Furthermore, it is clear that the BP180 ectodomain can not cover the entire spectrum of anti-BP180 antibodies present in these patients since reactivity with the intracellular domain of BP180 was demonstrated in about a third of BP patients (12,20).

For routine use of the Sf21-based eukaryotic assay in laboratories without access to molecular biological technology it is interesting to note that in principle, FL-BP180 expression can be conserved (at least to a large extent) when

infected Sf21 insect cells were fixed with acetone and stored at -20°C. Further improvement of the storage conditions is certainly required and should be addressed in the nearest future.

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References

1. Bernard P, Vaillant L, Labeille B, Bedane C, Arbeille B, Denoeux JP, Lorette G, Bonnetblanc JM, Prost C. Incidence and distribution of subepidermal autoimmune bullous skin diseases in three French regions. Arch Dermatol 1995; 131: 48-52
2. Zillikens D, Wever S, Roth A, Hashimoto T, Bröcker EB. Incidence of autoimmune subepidermal blistering dermatoses in a region of central Germany. Arch Dermatol 1995; 131: 957-958
3. Stanley JR, Hawley-Nelson P, Yuspa SH, Shevach EM, Katz SI. Characterization of bullous pemphigoid antigen: a unique basement membrane protein of stratified squamous epithelia. Cell 1981; 24: 897-903
4. Labib RS, Anhalt GJ, Patel HP, Mutasim DF, Diaz LA. Molecular heterogeneity of the bullous pemphigoid antigens as detected by immunoblotting. J Immunol 1986; 136: 1231-1235
5. Zillikens D, Giudice GJ. BP180/type XVII collagen: its role in acquired and inherited disorders or the dermal-epidermal junction. Arch Dermatol Res 1999; 291: 187-194
6. Borradori L, Sonnenberg A. Structure and function of hemidesmosomes: more than simple adhesion complexes. J Invest Dermatol 1999; 112: 411-418
7. Matsumura K, Amagai M, Nishikawa T, Hashimoto. The majority of bullous pemphigoid and herpes gestationis serum samples react with the NC16a domain of the 180-kDa bullous pemphigoid antigen. Arch Dermatol Res 1996; 288: 507-509
8. Zillikens D, Rose PA, Balding SD, Liu Z, Olague-Marchan M, Diaz LA, Giudice GJ. Tight clustering of extracellular BP180 epitopes recognized by bullous pemphigoid autoantibodies. J Invest Dermatol 1997; 109: 573-579
9. Zillikens D, Mascaro JM, Rose PA, Liu Z, Ewing SM, Caux F, Hoffmann RG, Diaz LA, Giudice GJ. A highly sensitive enzyme-linked immunosorbent assay for the

detection of circulating anti-BP180 autoantibodies in patients with bullous pemphigoid. J Invest Dermatol 1997; 109: 679-683
10. Giudice GJ, Emery DJ, Zelickson BD, Anhalt GJ, Liu Z, Diaz LA. Bullous pemphigoid and herpes gestationis autoantibodies recognize a common non-collagenous site on the BP180 ectodomain. J Immunol 1993; 151: 5742-5750
11. Egan CA, Taylor TB, Meyer LJ, Petersen MJ, Zone JJ. Bullous pemphigoid sera that contain antibodies to BPAg2 also contain antibodies to LABD97 that recognize epitopes distal to the NC16A domain. J Invest Dermatol 1999; 112: 148-152
12. Perriard J, Jaunin F, Favre B, Büdinger L, Hertl M, Saurat JH, Borradori L. IgG autoantibodies from bullous pemphigoid (BP) patients bind antigenic sites on both the extracellular and the intracellular domains of the BP antigen 180. J Invest Dermatol 1999; 112: 141-147
13. Liu Z, Diaz LA, Troy JL, Taylor AF, Emery DJ, Fairley JA, Giudice GJ. A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180. J Clin Invest 1993; 92: 2480-2488
14. Schmidt E, Obe K, Bröcker EB, Zillikens D. Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid. Arch Dermatol 2000; 136: 174-178
15. Kelly SE, Wojnarowska F. The use of chemically split tissue in the detection of circulating anti-basement membrane zone antibodies in bullous pemphigoid and cicatricial pemphigoid. Br J Dermatol 1988; 118: 31-40
16. Schmidt ES, Zillikens D. Autoimmune and inherited subepidermal blistering diseases: advances in the clinic and the laboratory. Adv Dermatol 2000; 16: 113-57
17. Balding SD, Prost C, Diaz LA, Bernard P, Bedane C, Aberdam D, Giudice GJ. Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain. J Invest Dermatol 1996; 106: 141-146
18. Nakatani C, Muramatsu T, Shirai T. Immunoreactivity of bullous pemphigoid (BP) autoantibodies against the NC16A and C-terminal domains of the 180 kDa BP antigen (BP180): immunoblot analysis and enzyme-linked immunosorbent assay using BP180 recombinant proteins. Br J Dermatol 1998; 139: 365-370
19. Husz S, Kiss M, Molnar K, Marczinovits I, Molnar J, Toth GK, Dobozy A. Development of a system for detection of circulating antibodies against hemidesmosomal proteins in patients with bullous pemphigoid. Arch Dermatol Res 2000; 292: 217-224
20. Kromminga A, Scheckenbach C, Georgi M, Hagel C, Arndt R, Christophers E, Bröcker EB, Zillikens D. Patients with bullous pemphigoid and linear IgA disease show a dual IgA and IgG autoimmune response to BP180. J Autoimmun 2000; 15: 293-300
21. Hirako Y, Usukura J, Uematsu J, Hashimoto T, Kitajima Y, Owaribe K. Cleavage of BP180, a 180-kDa bullous pemphigoid antigen, yields a 120-kDa collagenous extracellular polypeptide. J Biol Chem 1998; 273: 9711-9717
22. Pohla-Gubo G, Lazarova Z, Giudice GJ, Liebert M, Grassegger A, Hintner H, Yancey KB. Diminished expression of the extracellular domain of bullous pemphigoid antigen 2 (BPAG2) in the epidermal basement membrane of patients with

generalized atrophic benign epidermolysis bullosa. Exp Dermatol 1995; 4: 199-206
23. Schmidt E, Kromminga A, Mimietz S, Leinfelder U, Sitaru C, Bröcker EB, Zillikens D, Zimmermann U. A Highly Sensitive and Simple Assay for the Detection of Circulating Autoantibodies against Full-Length Bullous Pemphigoid Antigen 180. J Autoimmun 2002; 18: 299-309
24. Schumann H, Baetge J, Tasanen K, Wojnarowska F, Schäcke H, Zillikens D, Bruckner-Tuderman L. The shed ectodomain of collagen XVII/BP180 is targeted by autoantibodies in different blistering skin diseases. Am J Pathol 2000; 156: 685-695
25. Hata Y, Fujii Y, Tsunoda K, Amagai M. Production of the entire extracellular domain of BP180 (type XVII collagen) by baculovirus expression. Dermatol Sci 2000; 23: 183-190
26. Haase C, Büdinger L, Borradori L, Yee C, Merk HF, Yancey K, Hertl M. Detection of IgG autoantibodies in the sera of patients with bullous and gestational pemphigoid: ELISA studies utilizing a baculovirus-encoded form of bullous pemphigoid antigen 2. J Invest Dermatol 1998; 110: 282-286

Rapid flow cytometric differentiation of anti-platelet antibodies with microspheres

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Background

Allo- and autoantibodies against membrane structures of human platelets are involved in various types of immune thrombocytopenic disorders and can cause even life threatening bleeding. During the last decades a great variety of tests has been published with varying specificity. Most of these tests detect platelet-associated Ig (i.e. the presence of Ig on or within the platelet) without platelet-specific antibodies, but they cannot discriminate between specifically bound Ig and merely adsorbed immune complexes without a pathophysiological role (1,2). Substantial improvement resulted from the development of the widely accepted MAIPA (monoclonal antibody immobilization of platelet antigens) test (3). This test detects glycoprotein specific Ig selectively and is not hampered by immune complexes. However, MAIPA is time consuming and requires about 4-6x 10^7 platelets, which is a serious limitation for patients with very low platelet counts and for little children with small blood volumes. The goal of this study was to overcome some of the limitations of the MAIPA test by utilizing the advantages of flow cytometry.

Material and methods

Preparation of microspheres

Goat anti-mouse Ig labelled magnetic microspheres (Dynal A.S, Oslo, Norway), goat anti-mouse IgG labelled polymeric beads (Bangs Labs., Inc., Fishers, IN, USA) and QuantumPlex™ Beads, consisting of dyed polymeric beads designed for multiplexed assays and labelled with goat anti-mouse Ig (Bangs Labs.), were used for these assays. Beads were washed twice with phosphate buffered saline (PBS) containing 0.1% sodium azid and 1% bovine serum albumin (BSA). They were subsequently incubated with monoclonal mouse anti-human antibodies anti-CD41 (Dianova, Hamburg) or anti-CD49b (clone Gi9) or anti-β_2 microglobulin (both from Coulter-Immunotech, Marseille, France) and stored over night at 4°C and also up to several months without loss of activity.

Preparation of platelets

Platelets were prepared from platelet rich plasma of well typed blood donors by three washings with PBS/BSA (1500g / 4°C). Platelets were incubated for 1 hour at room temperature or over night at 4°C with human platelet specific antisera (anti-HPA-1a, anti-HPA-5b), with HLA-antisera (anti-Bw4 and anti-Bw6, Biotest, Dreieich, Germany) or with human AB serum as negative control.

Detection of auto-/alloantibodies

After incubation these platelets were washed and treated as follows. Platelets with bound antisera of patients were solubilized with 0.5% Triton X-100. Simultaneously, Dynal beads with mouse anti-human antibodies were added to these lysed platelets in order to bind to their corresponding ligands, i.e. the bimolecular complexes. This incubation took 30 minutes at 4°C on a rotating shaker. After immobilization of membrane molecules, magnetic microspheres were washed twice on a magnet particle concentrator (MPC) from Dynal with PBS/BSA supplemented with 0.5mM $CaCl_2$ and incubated with biotinylated goat anti-human IgG (H+L), F(ab')$_2$ fragment (Jackson, ImmunoResearch Laboratories, West Grove, PA, USA) for 30 minutes at 4°C on a rotating device in order to label the bound allo-/autoantibody. Beads were washed twice on a MPC and stained with Extravidin® R-Phycoerythrin conjugate (Sigma). After a

final washing, beads were resuspended in PBS and run on a flow cytometer. Most of the following results from different experiments are reported as multiples of mean fluorescence intensity of AB serum ('relative MFI').

Results

Fig. 1 showes typical examples of intact platelets, labelled with HLA-alloantibodies or AB serum (negative control) and anti-human IgG R-PE, and of microspheres with HLA-molecules from lysed platelets and anti-human IgG R-PE. Intact platelets exhibit a very broad standard deviation with a mean

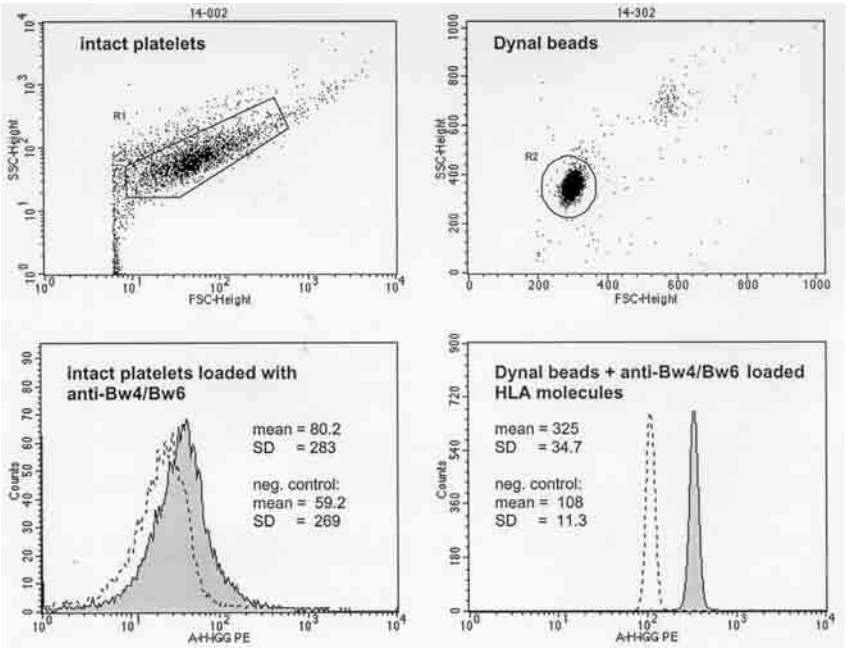

Fig. 1: Histograms of intact platelets loaded with alloantibodies and of HLA molecules of the same platelets after lysis and immobilization on Dynal beads.
Histograms from negative controls (AB serum) are presented by dashed lines.

value only slightly increased compared to the negative control. On the other hand, signals from lysed platelets are well separated.

How many platelets are necessary for one test?

Incubation of a constant amount of beads with increasing numbers of lysed platelets results in a linear increase of fluorescence signals (fig. 2), positive signals are obtained with less than 10^6 platelets if working with Dynal beads.

Titration of antiserum

Preincubation of constant numbers of platelets with increasing amounts of antiserum enhances signal strength (fig. 3). To compare the efficiency of Dynal beads with Bangs unstained beads and with QuantumPlex™ beads, several tests have been performed in one vial at the same time (multiplexed setting). Dynal beads give the strongest signals even with platelet antigens of low density (fig. 4).

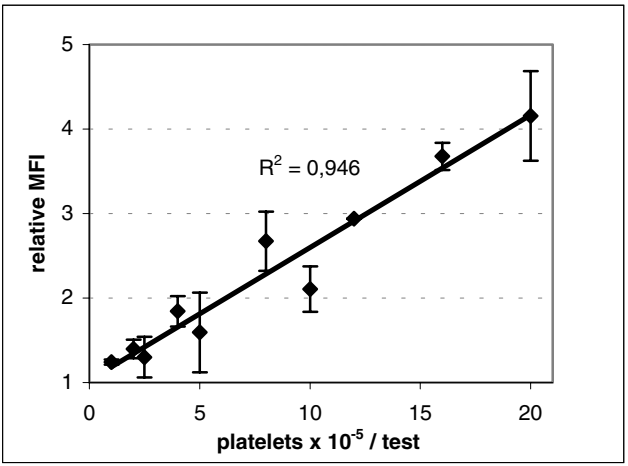

Fig. 2: How many platelets per test are necessary? Platelets preincubated with anti-HLA sera have been titrated down to 1x10^5/test before solubilization. If working with Dynal beads this is the minimal necessary amount of cells.

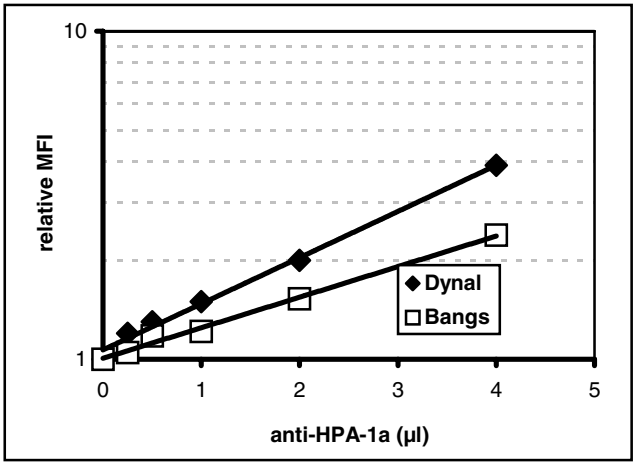

Fig. 3: Titration of antiserum. 2x10^6 platelets were preincubated with increasing amounts of antiserum, solubilized and bound to beads. Differences between both types of beads are significant (p = 0,043).

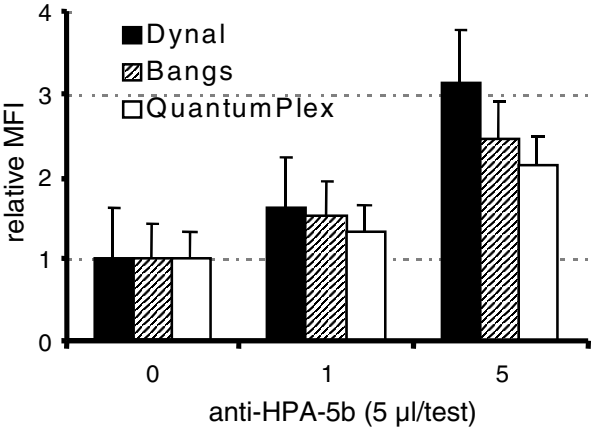

Fig. 4: Dynal beads produced the strongest signals in multiplexed experiments (mean of 6 experiments).
Signals of Dynal and Bangs beads are significantly stronger at 5µl of antiserum than signals of QuantumPlex™ beads.

Is multiplexed analysis a way to improve the test?

QuantumPlex™ beads offer the advantage of simultaneously testing several platelet antigens in a few microliters of patients serum, yielding, however, smaller signals than Dynal beads. Fig. 5 gives an example of multiplexed testing with QuantumPlex™ beads. Platelets incubated with a mixture of various antisera were washed and lysed. Five batches of presensitized beads specific for different platelet antigens were mixed in one vial and added to the lysed platelets. If platelet antigens carry autoantibodies, then the complex of beads, membrane molecules and autoantibodies will be positively stained with reporter antibody labelled with Phycoerythrin. As discussed above, fig. 4 showes another example of multiplexed testing.

What about specificity and cross reactivity?

To check the specificity and to exclude any cross reactivity of this assay, well characterized platelets were incubated with known single or mixed antisera and lysed. The glycoproteins gpIa/IIa were immobilized on QuantumPlex™

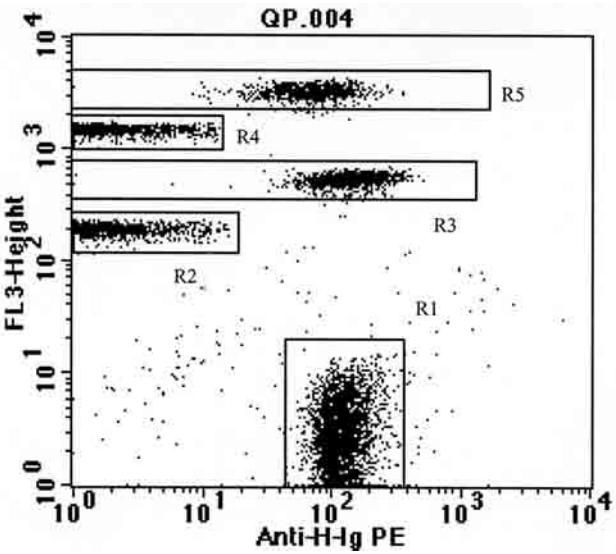

Fig. 5: Example of a multiplexed assay. Platelets were loaded with a mix of different antisera, solubilized and incubated with QuantumPlex™ beads.

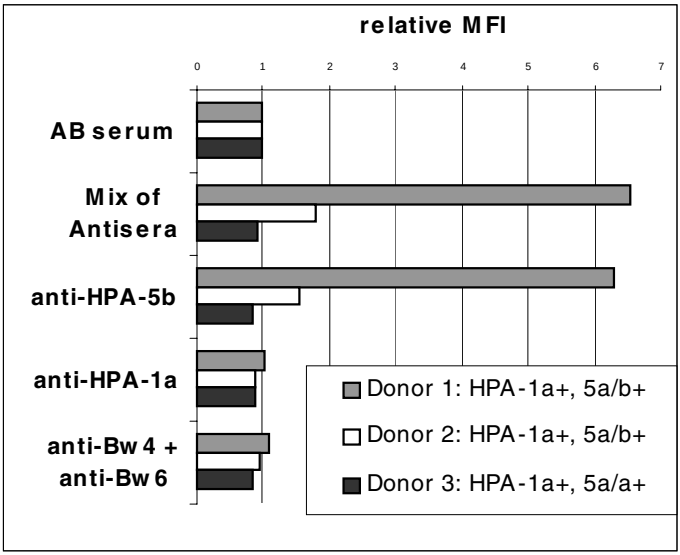

Fig. 6: Cross reactivity has not been observed.
Platelets of different donors were incubated with different antisera and lysed. The glycoproteins gpIa/IIa were immobilized on QuantumPlex™ beads presensitized with anti-CD49b.
Only samples with mixed antisera and with anti-HPA-5b give positive signals. Positive and negative signals are well separated.

beads which had been presensitized with anti-CD49b. As illustrated in fig. 6, positive and negative results are as expected and no cross reactivity has been observed. Specificity without cross reactivity was also obtained with beads presensitized with anti-CD41 (data not shown).

Discussion

The clinical importance of platelet specific allo- and autoantibodies, responsible for severe immune thrombocytopenias, requires a reliable and specific assay. Many published assays are not able to distinguish between specifically bound immunoglobulins and coating of membrane associated immune complexes on the surface of intact platelets, which why they can only be used as screening tests. Flow cytometry based tests on intact platelets have been pub-

lished, which quantify the amount of platelet associated Ig. They work fast and with as few as 10^5 to 10^6 platelets, but they are only of limited value (1,2). Further experiments with normal platelets, loaded over night with a mixture of anti-Bw4 and anti-Bw6 or with AB serum (data not shown), resulted in moderate, small or no differences between the mean values of specifically loaded platelets and negative controls and in very broad standard deviations of fluorescence signals (paper in preparation). These ambiguous and misleading results caused us to stop such experiments. On the other hand, signals from all of these specifically loaded platelets were unequivocally positive after solubilization with a very small standard deviation, as illustrated in fig. 1.

To date, the MAIPA test is well accepted as a routine method for detection of platelet specific antibodies. A three-molecular complex is immobilized and stained for quantitative determination with all other human immunoglobulines interfering with the test being washed out. However this test has some limitations, since it requires about 4-6 $x10^7$ platelets, and hands-on time is about one day.

We have combined lysis of platelets and immobilization of membrane molecules on the bead surface without a need for centrifugation. Obviously, Dynal beads have a low tendency for nonspecific binding, which does not increase during this incubation.

Experiments have been performed to study some important test variables. Using Dynal beads, the minimal amount of platelets required was found to be about 10^5 to 10^6 platelets per test. Increasing numbers of specifically loaded platelets produced a linear increase of fluorescence signals. (Bangs and QuantumPlex™ beads are slightly different, paper in preparation.) If platelets were incubated before solubilization with increasing amounts of antibody, relative MFI increased logarithmically with Dynal beads being superior to Bangs beads. Similar experiments with QuantumPlex™ beads and a different antiserum confirm these results and show that QuantumPlex™ beads yield the lowest signals. Additionally, these tests are examples of multiplexed testing, since three beads have been pooled, incubated, afterwards stained in one vial and run simultaneously on a flow cytometer. Multiplexing is possible with beads of different size or if they are prestained with different amounts of a fluorescence dye by the manufacturer, as done with Quantum-Plex™ beads (see fig. 5).

An important question to be addressed is the avoidance of any cross reactivity. Different well characterized antisera have been pooled and incubated with different well typed test platelets, yielding results according to expectation, as shown in fig. 6. Thus, this test system produces reliable and specific data without signs of cross reactivity.

Conclusion

The important features of this method are

- the small amount of platelets and reagents needed,
- reduction of hands-on time (the total test takes about 3-4 hours),
- signal amplification using the biotin-Extravidin® binding and R-Phycoerythrin as a very strong dye,
- reading the samples by flow cytometry,
- fast and effective washing procedures using magnetic beads,
- multiplexing capabilities,
- very few problems with nonspecific binding and
- multiple choices for the use of optimized surfaces (if compared with multiwell plates as used for MAIPA).

The most useful type of beads for this purpose would be magnetic beads which are prestained with a fluorescence dye of different strength to perform multiplexing, with high specific antibody binding capacity and low tendency to nonspecific binding of human proteins. However, such beads are not yet commercially available.

Acknowledgement

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References

1. Legler TJ, Humpe A, Leib U, Riggert J, Köhler M. Flow-cytometric analysis of platelet reactive allo- and autoantibodies. J Lab Med 1997; 21: 676-680
2. Janisiw M, Eichelberger B, Koren D, Panzer S. Screening for platelet autoantibodies by flow cytometry and their evaluation by the MAIPA technique. Wien Klin Wochenschr 1998; 110 (15): 531-4
3. Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood 1987; 70: 1722-1726