Early events in autoimmunity studied by antibody phage display

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Early events in autoimmunity studied by antibody phage display

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Cover illustration

“Untitled (Trapped)” by Paul Klee (1879–1940). Paul Klee, a prominent 20th century artist born in Bern (Switzerland), is probably the most famous person to suffer, and finally die from scleroderma. Scleroderma is one of the autoimmune diseases described in this thesis, and primarily affects the skin. “Untitled (Trapped)” is one of Klee’s last paintings, and represents the painter, trapped in the prison of his disease (visualized by the steel bars), which rendered him physically immobile.
Our environment is populated with a wide variety of micro-organisms, such as bacteria, viruses, and parasites, which can cause a broad range of diseases. Humans have developed a complex mechanism to fight against such pathogenic intruders: their immune system. Many different cells are involved in this defence, and each of these cells carry out specific jobs. An important step in the removal of pathogens is their recognition, a process in which the antibody-producing B cells are one of the key players. The theory of specific antibody formation was first introduced by Paul Ehrlich in 1900. In 1908, he and Ilya Mechnikov received a Nobel Prize in Physiology or Medicine for their work on immunity. From Ehrlich’s Nobel Lecture: “The relations between toxin and its antitoxin are strictly specific – tetanus antitoxin neutralizes exclusively tetanus toxin, diphtheria serum only diphtherial toxin, snake serum only snake venom, to mention just a few examples out of hundreds.” Their specificity makes antibodies very valuable for the characterization of proteins. In fact, antibodies do not react with the complete pathogen, but only with one or more antigenic determinants (epitopes) present on the pathogen. In autoimmune patients, autoantibodies are directed to molecules of the body itself. The specificity of autoantibodies can be advantageous for research and diagnostics, since many autoantibodies are associated with particular diseases. This chapter summarizes the current knowledge on antibody applications, with special focus on the antibody phage display technology, and provides an introduction to autoimmune diseases and their autoantibodies.

Antibodies in the immune system

As depicted in Figure 1a, the basic antibody structure is composed of four chains: two (identical) light chains and two (identical) heavy chains, which are linked by covalent binding through disulfide bonds, and by non-covalent bindings through hydrogen bonds and hydrophobic interactions (1). Similar interactions link one heavy- and light-chain pair with another (identical) pair. A major characteristic of immunoglobulins is their diversity. Remarkably, it has been estimated that lymphocytes are capable of producing \( 10^{15} \) different antibodies, whereas the diversity of the human immune repertoire originates from around 400 genes (2). In humans, diversity is generated through somatic recombination of variable (V), diversity (D) and joining (J) gene segments in the variable region of the heavy chain, and of
V and J segments in the variable region of the light chain, through pairing of heavy and light chains and through somatic mutations (3). The variable regions are involved in antigen binding, and are composed of four framework regions and three hyper-variable regions, which are called complementarity determining regions (CDRs) (4). The constant regions are involved in secondary effector functions, such as complement activation and binding to specific cell types (1).

Antibodies in the laboratory

Biological mechanisms, in health and disease, will be better understood when we know the function of the participating proteins (5,6). In such studies, antibodies are important tools, because they can be extremely helpful in functional studies, protein-protein interaction studies and localization studies (7).

Polyclonal and monoclonal antibodies

In general, polyclonal antibodies are produced by immunization with proteins, conjugated peptides (8), or DNA expression vectors (9), and specific antibodies can be isolated from the (anti)serum by affinity purification (10). The fact that a polyclonal antiserum recognizes multiple epitopes on one target is advantageous, because such a serum can be used in a wide variety of experiments (11). However, a drawback of a polyclonal serum is that blood withdrawal, even from the same animal, is unique and therefore not completely reproducible (11). Monoclonal antibodies (mAbs) are produced by a single B cell clone derived from animals immunized by similar procedures, and as a consequence they are directed to only one epitope (1). The two most common methods to generate monoclonal antibodies are by the hybridoma technology and by antibody phage display.

Monoclonal hybridoma technology

In 1975, Köhler and Milstein published the principles for the production of monoclonal antibodies, which is based on the fusion of a mouse myeloma cell line with spleen cells from an immunized mouse (12). For this discovery they received the Nobel prize in medicine in 1984. Remarkably, the hybridoma technology was never patented, and although the technique is laborious (5), monoclonal antibodies against numerous antigens have been generated with this technique (13). In addition to their use as important research tools, monoclonal antibodies have been successfully applied in therapy (14-16). At the end of 2005, eighteen monoclonal hybridoma antibodies have been approved for therapeutic applications, and several others are being tested in phase 2 and 3 clinical studies (17,18).

Two strategies have been used to reduce or prevent a human immune response to murine antibodies. One approach is the production of complete human monoclonal antibodies by immortalization of human B cell clones (19), but the establishment of this technique, and the production of sufficient amounts of human antibody from these cell lines appeared to be difficult (19,20). The other approach is to prepare chimeric antibodies by replacing the murine Fc region by a human sequence (21), or to ‘humanize’ murine antibodies by replacing the CDRs of a human antibody with the corresponding CDRs of the murine antibody of interest (22). Fifteen of the eighteen therapeutic monoclonal antibodies currently on the market are chimeric or humanized antibodies (18).

Antibody phage display technology

An alternative method to generate monoclonal antibodies is offered by the antibody phage display technology. Bacteriophages, in this chapter referred to as phages, are viruses that infect and propagate in bacteria (23). Phage display was first described by George Smith in 1985 (24), and is based on two crucial concepts (25). The first is that the gene encoding the peptide or protein that is displayed on the phage surface is contained within the phage particle, and that this linkage between genotype and phenotype is maintained when the phages replicate. The second is that large cDNA libraries can be cloned into the phages. Compared to the hybridoma technology, antibody phage display is easier, cheaper and less time-consuming (26). Due to the use of large naïve libraries, immunization can be by-passed (27). Other advantages of such large naïve libraries is that one library can be used for all antigens, and that antibodies can be isolated against antigens that are toxic or non-immunogenic in laboratory animals (28). Many variables have to be considered when preparing phage display libraries, including the display system (phage versus phagemid; in the phage system the encoding gene is inserted into the phage genome, whereas in the phagemid system the encoding gene is present on a plasmid), the coat protein that is used for fusion (in general either major coat protein VIII or minor coat protein III), and the (poly)peptide that is displayed (in case of antibody fragments scFv versus Fab; see Figure 1). The libraries used in the experiments described in this thesis are based on single chain variable fragment (scFv)
antibodies fused to minor coat protein III in a phagemid system. For extensive information on antibody phage display the reader is referred to a selected number of excellent reviews (23,26,29-31).

An scFv antibody fragment (see Figure 1c) consists of the two variable domains of an immunoglobulin molecule, namely the variable domain of the heavy chain (V\(_H\)) and the variable domain of the light chain (V\(_L\)), connected by a linker sequence (32), that enables proper folding of the antibody fragment. A schematic overview of the preparation of a library is presented in Figure 2.

Libraries can be prepared from immunized individuals (immune libraries) (33) and from naïve IgM antibody repertoires (naïve libraries) (28). Lymphocytes from blood, bone marrow, spleen, lymph nodes or tonsil can be used as source material to prepare immunoglobulin sources from spleen, lymph nodes or tonsil can be used as source material to prepare immunoglobulin libraries. Several synthetic libraries are available as well (34). In case of synthetic libraries, the antibodies are built artificially by in vitro assembly of synthesized V- and D-/J- gene segments. A higher diversity is obtained by introducing a pre-determined level of randomization of the CDRs. Figure 3 illustrates a general procedure for the selection of antibodies from phage display libraries. After one or more selection rounds, monoclonal phages are analyzed for reactivity with the target antigen. Often, robotic equipment is used in the laborious parts of the selection procedure (35,36).

**Target discovery by antibody phage display**

Proteins or peptides that are only expressed on specific cells or tissues, or under certain (pathological) conditions, are potential targets for therapeutics and diagnostics. During the last decade, many antibody phage display approaches have been developed for the identification of such biomarkers, and most of these include a subtraction or competition step to remove phages that bind to ‘common’ or non-specific epitopes (31). As schematically shown in Figure 4, such selection strategies end up with antibodies to unknown cell- or tissue-specific targets, which can subsequently be purified using the antibody as bait, and identified by mass spectrometry. A major advantage of this procedure is that when biomarkers are discovered, at the same time antibodies to these molecules are available.

Monoclonal antibodies to biomarkers obtained by these recombinant DNA technologies (recombinant antibodies) have been engineered for (tumour) imaging, chemotherapy, immunotherapy, and radio-immunotherapy. At this moment, one recombinant phage display-derived antibody, Adalimumab (trade name Humira), is approved for therapy of psoriatic arthritis and rheumatoid arthritis, and many more are in clinical and pre-clinical trials (31).

Figure 3 provides a schematic overview of the most common methods for the identification of biomarkers using antibody phage display. The most straightforward method to discover novel (tumor) cell surface markers is direct selection of antibody phage libraries on intact cells (Figure 5a) (37-43). In 1995, de Kruif et al. were the first to use mouse antibodies to known cell surface markers to isolate phages directed to unknown markers on the same cell (44). They incubated a large synthetic antibody library with a peripheral blood leukocyte preparation, in which CD3+ and CD20+ cells were fluorescently labelled with specific antibodies, and sorted the labelled cells by fluorescence activated cell sorting (FACS). The isolated phages bound to the CD3+ and CD20+ cells, and to a limited set of other cells. This approach has been effectively applied in a number of studies (45-50). Cell surface markers have also been identified by in vivo or ex vivo selections (Figure 5e). For example, scFvs binding to thymic cells have been successfully identified by isolating phages from the thymuses of mice that were injected intravenously with an antibody phage.
library (51). Theoretically, ex vivo selections should be applicable for antibody libraries, but so far they have only been described for peptide libraries (52).

An elegant method for the identification of both extracellular and intracellular antigens involves panning of phage libraries on tissue cryosections, followed by the isolation of specific cells or pieces of tissue by laser microdissection (Figure 5d) (53).

Identification of intracellular markers by subtractive antibody phage display is mainly based on the use of cellular extracts (Figure 5 b, c, and f). Cellular extracts can be immobilized in many ways, for example by (i) coating on immunotubes or the wells of microtiter plates (54-56), by (ii) blotting to membranes after electrophoresis (57) or directly by dot blotting (58,59), and by (iii) biotinylation in combination with streptavidin-coated beads (60,61).

Finally, two studies describe the isolation of differentially expressed proteins, visualized by two-dimensional gel electrophoresis, prior to selection. Pini et al. excised specific protein spots from two-dimensional gels, and subsequently eluted the proteins from the gel slices. The proteins were biotinylated and used for selection of specific phages (60). Liu et al. used a similar approach, in which they excised differentially expressed protein spots from two-dimensional western blots and used these spots for screening of phage display antibody libraries (62).

Figure 5. Overview of subtractive antibody phage display approaches to discover proteome-specific target epitopes, with subtraction or competition to remove binders to common epitopes. (a) Selection on cells. Phages are incubated with a cell population, and specific cell types are isolated by labelled antibodies and FACS. The phages binding these selected cells can be isolated. (b) Selection on coated cell extracts. Cell extracts with target epitopes are immobilized on immunotubes, and phages recognizing common epitopes are subtracted by competition with non-immobilized cell extract. (c) Selection on biotinylated cell extracts. Cell extracts, containing target epitopes, are biotinylated for capture with magnetic streptavidin-coated beads, whereas non-biotinylated proteins with common epitopes can be used for competition. (d) Selection on tissue sections. Antibody phage display libraries are incubated with tissue sections and areas containing specific cells or structures are excised by means of laser microdissection. (e) In vivo selections. Phage libraries are injected in laboratory animals. Phages can then be isolated from specific organs or parts of the body. (f) Selection on blotting membranes. Antibody phage display libraries are incubated with blotting membranes, containing proteins from cell extracts, optionally separated by two-dimensional gel electrophoresis.

Antibodies in autoimmune diseases: autoantibodies

Autoimmune diseases, affecting approximately 2-3% of the population of the industrialized world (63), can be divided into systemic and organ-specific autoimmune diseases. The autoimmune diseases studied in this thesis are systemic autoimmune diseases, and include systemic sclerosis (SSc), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), polymyositis/dermatomyositis (PM/DM), and SLE overlap disease (also referred to as mixed connective tissue disease, MCTD). An overview of the characteristics of these diseases is presented.
in Table 1. A hallmark of these diseases is that patients produce high titres of antibodies (autoantibodies) directed to specific “self” molecules (autoantigens).

It is still unclear why autoimmune patients produce autoantibodies. The autoimmune response is a complex process, of which the underlying mechanisms are still poorly understood. It is believed that (a combination of) genetic, hormonal, and environmental factors are involved. A model that is increasingly supported by experimental data, hypothesizes that unusual posttranslational modifications play an important role in breaking tolerance to self-antigens (see reviews by (64-67)). It has been shown that dying cells represent an important source of such unusual protein modifications. During apoptosis and necrosis many known autoantigens are modified, as for example by proteolytic cleavage, hyper- or dephosphorylation, or citrullination (65-68). Furthermore, oxidative fragmentation of proteins has been put forward as a mechanism of autoantigen modification (69,70). It is believed that modified autoantigens contain neo-epitopes that, upon exposure to the immune system, trigger a primary immune response. Subsequent responses may lead to the generation of autoreactivity to other, not modified parts of the antigen via a process called epitope spreading. In this process, the secondary immune responses may spread intramolecularly, but also intermolecularly to other proteins that are stably associated with the primary target (71). Data supporting this idea mainly result from experiments on the U1-70K protein, a major autoantigen in SLE overlap disease, and will be discussed in detail in Chapter 5.

Autoantibodies are in some cases specifically associated with a certain autoimmune disease. For example, anti-citrullinated protein autoantibodies (ACPA) are almost exclusively found in patients suffering from rheumatoid arthritis (reviewed by (72)), and autoantibodies directed to the U1 small nuclear ribonucleoprotein (snRNP) complex are associated with SLE overlap syndrome (reviewed by (73)). Autoantibodies can be detected in patient sera with a number of laboratory tests, and the results are in most cases helpful for rheumatologists to diagnose patients and to provide prognoses. Autoantibodies to cell death-specific epitopes are believed to be present in the beginning of disease, and identification of such ‘early’ targets might therefore allow an earlier diagnosis and treatment of autoimmune diseases.

**Outline of the thesis**

The goals of this research project were to develop novel applications of the antibody phage display technique, and to use these applications to gain more insight into the occurrence of autoantibodies and their antigenic targets.

The results described in this thesis can be divided into two parts:

**Part 1:** Novel applications of the antibody phage display technology

**Part 2:** Autoantigenicity of cell death-associated epitopes

In Part 1, novel antibody phage display applications are described. Chapter 2 illustrates an approach to reduce the number of animals needed for antibody phage display experiments. Libraries were prepared from chickens that were simultaneously immunized with multiple antigens, the majority of them being human autoantigens, and antibodies recognizing the individual antigens were isolated from these libraries. In Chapter 3 it is demonstrated that, by fusing constant domains from immunoglobulins from different organisms (human, mouse and chicken), the recombinant antibodies become attractive tools for double- and triple-staining of cells and tissues. Chapter 4 describes a novel subtractive phage display approach, which is applicable for the identification of disease-related proteins that are specifically present in a certain cell type or tissue, or specifically expressed under certain conditions. The approach combines repeated subtractive selection in solution, followed by selection on western blots of two-dimensional gels. This approach allowed the identification of apoptosis-specific epitopes that are targets of autoantibodies in systemic autoimmune diseases.

In Part 2, the antigenic targets, identified by the subtractive phage display experiments in Chapter 4, are further characterized. These apoptosis-specific epitopes are present on the proteins U1-70K, hnRNP C, and p54<sub>ub</sub>/PSF. Chapter 5 elaborates on the involvement of neo-epitopes in breaking tolerance to self-antigens. Focus is on the modifications of the U1 snRNP complex, a major autoantigen in SLE overlap syndrome (also named mixed connective tissue disease, MCTD).
In Chapter 6, patient sera are analyzed for reactivity with apoptotic and non-apoptotic U1-70K, and it is demonstrated that the apoptotic form of U1-70K is more autoantigenic compared to its non-apoptotic form. Furthermore, apoptotic U1-70K is shown to be recognized by patient sera early in the disease, which provides additional evidence that apoptotic modifications might play a role in breaking tolerance to self-antigens. The autoantigenicity of hnRNPs C and p54\textsuperscript{HH/PSF} is described in Chapter 7. Finally, the antigenicity of epitopes generated by oxidative fragmentation is described in Chapter 8.

In Chapter 9, the results obtained in this thesis work are discussed as well as possible implications for further research.

References

Part I

Novel applications of the antibody phage display technology
Multiple-antigen immunization of chickens facilitates the generation of recombinant antibodies to autoantigens

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Multiple-antigen immunization in chicken

Antibody phage display is a powerful tool for the generation of monoclonal antibodies against virtually any given antigen. Chickens are phylogenetically more distant from humans compared to other laboratory animals like mice and rats. Therefore, the use of chickens is especially beneficial when generating recombinant antibodies against human autoantigens, which are often highly conserved among mammals. Another advantage of using chickens in antibody phage display is that the preparation of scFv antibody libraries is faster and easier compared to preparing such libraries from other species, since only limited primer sets are needed for amplification of the chicken V_h and V_L genes. In the present study we explored the possibility to immunize chickens with antigen cocktails for the generation of recombinant antibody fragments directed to a range of human autoantigens. After immunization of chickens with two cocktails of 7 recombinant, bacterially expressed autoantigenic proteins, the polyclonal chicken sera reacted strongly with most of the antigens used for immunisation. By creating and screening single-chain variably fragment antibody phage display libraries, recombinant monoclonal antibody fragments were successfully isolated against the autoantigens annexin XI, CENP-B, HspB3, DNA topoisomerase I, Jo-1, Ro52, Ro60, Rpp30, and U1A. In conclusion, the immunization of only four chickens with two distinct pools of in total 14 autoantigenic proteins allowed the isolation of scFvs against 9 of these antigens.

Introduction

Autoimmune diseases are characterized by the presence of serum antibodies (autoantibodies) directed to ‘self’-antigens (autoantigens). Characterization of autoantibodies and their targets will provide more insight into the underlying mechanisms of autoimmune diseases. However, characterization of autoantigens in for example tissue sections of patient biopsies or on western blots containing human patient material (such as serum) is not possible with patient autoantibodies, due to cross-reaction of the secondary antibodies with human immunoglobulins present in the tissue or serum. This problem can be circumvented by using antibodies from other species. Antibody phage display has brought a revolution in the preparation of monoclonal antibodies, since it is easier, faster and less expensive compared to conventional hybridoma technology. The phage display technique is based on the display of (poly)peptides, such as antibody fragments, on the surface of bacteriophages (1). Recombinant antibodies have been selected from immune libraries, naïve libraries (2) and (semi-)synthetic

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libraries (3). One of the advantages of immune libraries is that clonal selection and affinity maturation in the individual generally lead to scFvs with higher affinities compared to scFvs isolated from naïve or synthetic libraries (4).

Recombinant antibodies to human autoantigens have been successfully isolated from patient-derived libraries (5-7). The disadvantages of using patient libraries are that the preparation of human libraries is relatively time-consuming, and furthermore that autoimmune patients generally produce autoantibodies to a small subset of autoantigens. This means that libraries from several patients have to be prepared to isolate antibody fragments to a range of autoantigens. Recombinant antibody fragments can also be isolated from animals immunized with human targets. Often mice are used for the preparation of immune libraries, but other animals have been used successfully as well, especially due to unique features of their immune systems. For example, the camelds produce next to complete immunoglobulins, homodimers of only heavy chains, which are devoid of light chains (8). These single domain llama V_{H} heavy chains have appeared to be highly soluble and very stable when expressed in bacteria (9). Another example is the chicken immune system, which is particularly beneficial when constructing libraries. Where mammals diversify their immune response through somatic V(D)J recombination, chickens use only one recombination event, in combination with a process called ‘gene conversion’ (10). In this process, pseudogenes, which are located upstream, are translocated into the heavy and light chain variable regions. Consequently, only two primer sets (one for the amplification of the V_{H} gene and one for the amplification of the V_{L} gene) are needed to construct chicken libraries (11). As a result, construction of chicken antibody libraries is much easier and faster compared to human and murine libraries. The advantage of using chickens is furthermore attributed by the fact that non-mammal species are more likely to evoke an immune response to epitopes that are highly conserved between mammals (12).

Here, we studied the immunization of chickens with two mixtures of seven distinct human autoantigens each, the preparation of scFv phage display libraries using material from these immunized animals, and the isolation of recombinant antibodies to these autoantigens. As a result of the selection procedures presented here, monoclonal scFvs to most of the antigens were obtained, which are available to be used for characterization of autoantigens in patient samples.

**Results**

**Immunization and generation of libraries**

A schematic overview of the library preparation and selection strategy is depicted in Figure 1. Four chickens were immunized with two distinct protein cocktails, which were composed of proteins that are autoantigenic in human (except HspB3), and which are in general highly conserved molecules with key functions in the cell. An overview of the characteristics of the antigens used is presented in Table 1. Prior to immunization, recombinant proteins were expressed in E. coli, and proteins are purified. The library with a long linker and the library with a short linker are pooled and selected on the individual antigens.
cocktails were prepared by mixing equal amounts of each purified protein. Pre-immune and immune sera were tested in ELISA for reactivity with the individual antigens and BSA as a negative control antigen. Since Rpp38 and hPop1 were applied as GST fusion proteins, reactivity to GST was measured as well. Figure 2 demonstrates the reactivities of the pre-immune sera, and of the sera obtained after the final immunization. None of the pre-immune sera showed significant reactivity with any of the antigens. Immune sera from chickens 1 and 2 showed strong reactivity with annexin XI, CENP-B, Topo, Jo-1, Ro60, U1A, and GST, but no or hardly any reactivity with Rpp38. Immune sera from chickens 3 and 4 reacted strongly with hPop1, Ro52, Rpp30, and GST, and did not or only very weakly react with α-fodrin, HspB3, Rrp4, and Rrp42. After immunization, antibody phage display libraries were constructed with a short linker of seven amino acids (GQSSRSS), and

Table 1  Antigen characteristics

<table>
<thead>
<tr>
<th>Autoantigen</th>
<th>Molecular weight (kDa)</th>
<th>Localization</th>
<th>Function</th>
<th>Antigen characteristics</th>
<th>References</th>
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</thead>
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<tr>
<td>Annexin XI</td>
<td>56</td>
<td>Cytoplasm and nucleoplasm</td>
<td>Phospholipid binding</td>
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<td>CENP-B</td>
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<td>DNA-binding</td>
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<td>115</td>
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<td>α-Fodrin</td>
<td>100</td>
<td>Nucleolus</td>
<td>DNA unwinding</td>
<td>DNA unwinding</td>
<td>(39)</td>
</tr>
<tr>
<td>Topo</td>
<td>170</td>
<td>Nucleoplasm</td>
<td>Small heat shock protein (dispenser)</td>
<td>Small heat shock protein (dispenser)</td>
<td>(39)</td>
</tr>
<tr>
<td>HspB3</td>
<td>240 / 120</td>
<td>Cytoplasm, nucleoplasm</td>
<td>Histidyl-tRNA synthetase (translation)</td>
<td>Histidyl-tRNA synthetase (translation)</td>
<td>(39)</td>
</tr>
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<td>Jo-1</td>
<td>30</td>
<td>Nucleolus</td>
<td>Component of RNase P / MRP</td>
<td>Component of RNase P / MRP</td>
<td>(39)</td>
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<td>Ro52</td>
<td>30</td>
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<td>Component of RNase P / MRP</td>
<td>Component of RNase P / MRP</td>
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<td>(41)</td>
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<td>Nucleoplasm</td>
<td>Component of the U1 snRNP</td>
<td>Component of the U1 snRNP</td>
<td>(41)</td>
</tr>
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Figure 2. Chicken serum ELISA. Chicken sera before immunization (pre-immune) and after the final immunization (immune) were analyzed for reactivity with the individual antigens in ELISA. Antigens were coated, incubated with the chicken sera at a 500-fold dilution, and bound antibodies were detected with HRP-labelled goat anti-chicken antibodies. After TMB conversion the absorbance was measured at 450 nm. Chicken sera were tested for reactivity with the antigens they were immunized with, and for reactivity with BSA as control antigen. Reactivity with GST was measured as well, since hPop1 and Rpp38 were GST fusion proteins. (a) Pre-immune and immune sera of chickens 1 and 2 were tested for reactivity with annexin XI, CENP-B, Topo, Jo-1, Ro60, U1A, GST and BSA. (b) Pre-immune and immune sera of chickens 3 and 4 were tested for binding to α-fodrin, hPop1, HspB3, Ro52, Rpp30, hRrp4, hRrp42, GST and BSA.
with a long linker of eighteen amino acids (GQSSRSGGGGSGGGGSGSGGGS), similar to the linkers described by Andris-Widhopf et al. (13). Subsequently, the libraries with short and long linkers from chickens immunized with the same antigen cocktail were pooled, and panned on the individual antigens. The complexities of the combined libraries were 2.1×10⁵ for the library corresponding to antigen mixture 1, and 1.1×10⁵ for the library corresponding to antigen mixture 2. Several selections were performed, with the main difference being the amount of antigen coated to the well and the number of selection rounds, as described in the Materials and Methods section. After selection, polyclonal phages of each selection round were tested in ELISA for reactivity with the antigen the pool was selected on. Figure 3 demonstrates ELISA results for the phages of the different selection rounds, in which the libraries were panned on annexin XI, α-fodrin, hPop1, Ro52, Rpp30, Rrp42, and U1A (polyclonal phage ELISA results of the other selections are not shown). The polyclonal phages were assayed for reactivity with the antigen the phages were selected on, and for “background” reactivity with the unrelated protein BSA. The polyclonal phage pools were detectably enriched for phages directed to annexin XI, Ro52, and U1A from the third rounds onwards. After the first four selection rounds, the polyclonal phage pool panned on hPop1 did not show reactivity with hPop1. After the fifth selection round a strong reactivity to hPop1 was observed, although this reactivity decreased in subsequent selection rounds.

Figure 3. Polyclonal phage ELISA. Libraries were selected on annexin XI, α-fodrin, hPop1, Ro52, Rpp30, Rrp42, and U1A, and subsequently analyzed for reactivity with these antigens in ELISA. Seven selection rounds were carried out and the reactivities of polyclonal phages after each selection round are measured. Numbers corresponding to the selection rounds are on the right. Bound phages were detected with mouse anti-M13 antibodies, in combination with HRP-labelled rabbit anti-mouse antibodies, followed by TMB conversion. The absorbance was measured at 450 nm.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen-specific phages</th>
<th>Cross-reactive phages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin XI</td>
<td>ASA2*, ASE2, ASE12*, AS6, ASG9</td>
<td>-</td>
</tr>
<tr>
<td>CENP-B</td>
<td>SD1, SD2, SD4</td>
<td>-</td>
</tr>
<tr>
<td>HspB3</td>
<td>6E5, 7B4, 7B9, 7B11, 7C5 (cross-reactive with Ro60)</td>
<td>-</td>
</tr>
<tr>
<td>Topo</td>
<td>SA12, SC2, SC3, SC6, SC8, SC9, SD6</td>
<td>-</td>
</tr>
<tr>
<td>Jo-1</td>
<td>SB4, SB7**, SB6, SB9, SB10**, SB11</td>
<td>-</td>
</tr>
<tr>
<td>Ro52</td>
<td>R1E4, R3G10, R2A11, R2E5, R2E6, R4F11, R2G8 (cross-reactive with Topo)</td>
<td>-</td>
</tr>
<tr>
<td>Ro60</td>
<td>-</td>
<td>5E2, SH10 (both cross-reactive with U1A)</td>
</tr>
<tr>
<td>Rpp30</td>
<td>SC9**, SD11, 6E12**</td>
<td>-</td>
</tr>
<tr>
<td>U1A</td>
<td>U1I8, U1I11***, U1I3, U1I15, U1I16, U1I18, U1I10, U1I4, U1I11, U1I8, U1I3, U1I12***, U1I1A2 (cross-reactive with annexin XI)</td>
<td>-</td>
</tr>
</tbody>
</table>

The V_h and V_l chains of underlined clones were sequenced. Anti-CENP-B clone SD4 and anti-Ro60 clone SH10 contained a long linker, all other clones contained short linkers. * Anti-annexin XI clones ASA3 and ASE12 differ one amino acid in CDR2 of V_h chain (V_l chains are identical) ** Anti-Rpp30 clones SC9 and 6E12 differ one amino acid in CDR2 of V_l chain (V_h chains are identical) *** Anti-Jo-1 clones SB6, SB9, and SB10 have different V_h chains. The V_l chains of SB6 and SB9 are identical, the V_l chain of SB10 differs three amino acids in CDR3 compared to SB6 and SB9. **** Anti-U1A clones U1I1B1 and U1I3D12 have identical V_h chains, and different V_l chains.

Table 2. Overview of isolated phages with antigen-binding activity

Screening of phages

After the analysis of polyclonal phages in ELISA, individual colonies were isolated to analyze their specificity in a monoclonal phage ELISA. Colonies were picked from the first, or first two selection rounds with high polyclonal phage ELISA reactivity. For each antigen, 96 to 144 monoclonal phages were first screened for full-length scFv cDNA insert by PCR. Subsequently, DNA fingerprint patterns were analyzed using the restriction enzyme BstNI, and phages were grouped based on fingerprint pattern similarities. From each fingerprint group, several phages were tested in ELISA for binding to their target antigens, and for potential cross-reactivity with the other proteins used for immunization. As summarized in Table 2, for most of the antigens (annexin XI, CENP-B, HspB3, Topo, Jo-1, Ro52, Rpp30, and U1A) several monoclonal phages were reactive in ELISA. Phages directed to HspB3 reacted weakly, but exclusively with their target antigen. Some phages cross-reacted very specifically with one of the other antigens. For example, anti-U1A phage U1I1A3 cross-reacted only with annexin XI in ELISA. Furthermore, two phages that were derived from selection on Ro60, reacted strongly with both Ro60 and U1A in ELISA, but no phages were detected that reacted with Ro60 exclusively. None of the selected monoclonal phages reacted detectably with α-fodrin, hPop1, Rpp30, Rrp42 and Rrp42 in ELISA.
Characterization of selected antibodies

After the first analysis with recombinant, purified antigens, we investigated the reactivity of the monoclonal phages with their eukaryotic antigens, which are present in a complex mixture of proteins. Therefore, the reactivity of a selected panel of monoclonal phages was analyzed on immunoblots containing a HeLa cell extract. As demonstrated in Figure 4, monoclonal phages A5A3 (anti-annexin XI, 56 kDa), 5B5 and 5B10 (anti-Jo-1, 50 kDa), U11B3, U13D12 and U11A3 (anti-U1A, 38 kDa), and 5D1 (anti-CENP-B, 80 kDa) reacted with proteins with molecular masses corresponding to those of their target antigens. Antisera with known reactivities towards the antigen of interest were used in parallel. Anti-U1A monoclonal phage U11A3, which was reactive with U1A and annexin XI in ELISA, reacted with proteins with molecular weights corresponding to U1A and annexin XI on western blot as well. Moreover, U11A3 also reacted with U2B’, which is structurally similar to U1A and which is also recognized by the anti-U1A monoclonal mouse antibody, and with another (so far unidentified) protein of approximately 50 kDa.

To shed more light on the molecular identity of the antibody fragments, the cDNAs of 24 randomly chosen, antigen reactive scFvs were sequenced. The sequenced clones are underlined in Table 2. Several anti-Jo-1 clones (5B6, 5B9 and 5B10) appeared to have (almost) identical V\textsubscript{H} chains, but different V\textsubscript{L} chains. The same holds true for anti-U1A clones U11B1 and U13D12. Furthermore, 22 clones (92%) contained a short linker, whereas only 2 clones (8%) contained a long linker, and this finding suggests that antibody fragments with a short linker are more easily selected in this approach compared to antibody fragments with a long linker.

Figure 4. Phage immunoblot analysis. HeLa cell extracts were prepared by sonication and western blotted onto nitrocellulose membranes. Phages were immunoblotted at a concentration of 5x10\textsuperscript{11} cfu/ml, and bound phages were detected with HRP-labelled mouse anti-M13 antibodies, followed by ECL. Reactivities of phages and control antisera against annexin XI (56 kDa, lanes 1 and 2), Jo-1 (50 kDa, lanes 3-5), U1A (38 kDa, lanes 6-9), and CENP-B (80 kDa, lanes 10 and 11) are shown. Molecular weights (kDa) of marker proteins are indicated on the left.

Discussion

Chickens are, next to mice and rabbits, an excellent source for the generation of antibodies. Chickens are phylogenetically more distant from humans, and as a consequence, conserved mammalian proteins are often more immunogenic in avian species than in mammals (12). Polyclonal antibodies (IgG-like antibodies termed IgY) are easily isolated from chicken egg yolk (14), and the generation of monoclonal chicken antibodies by the hybridoma technology has been described (15), although these techniques are not routinely used (14). In a number of studies, recombinant monoclonal antibodies have been successfully isolated from chicken libraries (11,13,16). The demonstration by Tsurushita et al. (17) that chicken antibodies can be readily humanized recently boosted the potential use of recombinant chicken antibodies for therapeutic applications.

Recently, Finlay et al. isolated recombinant antibodies directed to two individual proteins, and yellow jacket venom, from a library that was prepared from a chicken immunized with a mixture of these proteins (18). Our study extends these observations and shows that chickens can be successfully immunized with a mixture of at least 7 distinct proteins. It also emphasizes the ease of using chickens for the generation of recombinant antibodies to highly conserved, human targets (such as autoantigens), and suggests a general applicability for antibody generation with a range of antigens. Immunization of animals with multiple antigens has been described previously for rabbits as well (19), and in this case scFvs against four haptens were isolated from a single immunized rabbit. Although the construction of rabbit libraries is easier compared to human or murine libraries, construction of chicken libraries is even more straightforward, since it involves only two primer sets. This makes chickens a very suitable alternative with respect to time and costs.

Phages were isolated against nine of the fourteen antigens used for immunization (annexin XI, CENP-B, HspB3, Topo, Jo-1, Ro52, R060, Rpp30, and U1A), and for most of the antigens more than one reactive phage was isolated. Phages directed to the other five antigens (α-fodrin, hPop1, Rpp38, Rrp4, and Rrp42) were not selected. This might be explained by the fact that the immune sera after the final booster reacted only weakly, or not at all, with these antigens, indicating that the chickens did not have an immune response towards these antigens. These findings suggest that the immune response was mainly directed to a panel of immunodominant epitopes present on the other antigens. The specific cross-reactivity of several monoclonal phages suggests that a similar epitope on two or more proteins is relatively immunogenic in chickens. This cross-reactivity could be explained by the fact that the process of gene conversion can occur multiple times in the life of a single B cell (see review by (20)). In case a B cell, producing antibodies to a first antigen, encounters a second (different) antigen during a subsequent immunization, it could be imagined that through gene conversion, the antibody reactivity slightly changes and that both antigens are targeted by this specific B cell.
The monoclonal phages isolated in this study, were shown to be reactive with their recombinant target antigen in ELISA, and several phages were also reactive with their counterparts expressed in a human cell. These phages are particularly useful for detection of autoantigens in human patient samples. We recently described a method in which scFvs are fused with constant domains from immunoglobulin light chains from different species, thereby enabling easy double- or triple-staining of antigens (21). Using these constant domain tags, the recombinant antibodies obtained in the study presented here can be easily used for simultaneous detection of the different autoantigens as well. The fact that some phages only reacted with recombinant protein, and not with the mammalian counterparts, may be explained by the difference in folding, or the presence of post-translational modifications that might lead to a change in the epitopes recognized by the phage antibody. In addition, the concentration of a particular protein in a HeLa cell extract, and thus the amount of this protein on the western blots, might be too low for detection.

It has been described that recombinant antibody fragments with linkers of more than twelve amino acids are predominantly monomeric, whereas scFv fragments with linkers of more than twelve amino acids are predominantly antibody fragments with linkers of more than twelve amino acids are predominantly multimeric or tetramers (22). It is thought that, during phage amplification, these diabodies are formed on the phage particle by association of the scFv fragment that is displayed on the phage with a soluble scFv fragment that is present as a result of proteolytic cleavage.

In conclusion, the immunization of only four chickens with two distinct pools of in total 14 autoantigenic proteins allowed the isolation of high avidity scFv’s against 9 of these antigens.

**Materials and Methods**

**Immunization and library construction**

The recombinant, purified proteins annexin XI (24), centromere protein B (CENP-B) (25), α-fodrin (26), human Pop1 protein (hPop1) (27), DNA Topoisomerase I (Topo) (28), heat shock protein R3 (HspR3) (29), histidyl tRNA synthetase (Jo-1) (30), Ro52 (31), Ro60 (32), Rpp30 (33), Rpp38 (33), Rrp4 (34), Rpp42 (34), and U1A (35), were available at the Department of Biochemistry, Radboud University Nijmegen, The Netherlands. hPop1 and Rpp38 were produced as glutathione S-transferase (GST) fusion proteins. Two different antigen cocktails were used for chicken immunization. Antigen cocktail 1 included annexin XI, CENP-B, Topo, Jo-1, Ro60, Rpp38, and U1A, whereas antigen cocktail 2 consisted of α-fodrin, hPop1, HspR3, Ro52, Rpp30, Rrp4, and Rpp42. Two couples of Barnevelder chickens were each immunized with antigen cocktail 1 and 2 respectively. The primary immunization was applied in complete Freund’s adjuvant, whereas following boosters were given in incomplete Freund’s adjuvant. ScFv phage display libraries were constructed with a short and a long linker as described previously (36). Shortly, V\textsubscript{H} genes were amplified using forward primer CSCVHo-F for a short linker, or forward primer CSCVHo-FL for a long linker, and reverse primer CSCG-B. V\textsubscript{L} genes were amplified with forward primer CSCVK and reverse primer CKJo-B. Amplified V\textsubscript{H} and V\textsubscript{L} genes were fused by overlap PCR using primers CSCVHo-F and CSCK-V. All primers used for library construction are listed in Table 3. Subsequently, V\textsubscript{H}-V\textsubscript{L} fragments were inserted in the pCombi vector through Sfi digestion and ligation, and transferred into E.coli TG1 by electroporation.

**Phage selection**

Phages were amplified from bacterial libraries as described previously (2). Isolated phages were resuspended in PBS containing 1% bovine serum albumin (BSA). Libraries A (antigen mixture 1, long linker) and B (antigen mixture 1, short linker) were pooled, and libraries C (antigen mixture 2, long linker) and D (antigen mixture 2, short linker) were pooled. Libraries were panned on the individual antigens, which were immobilized on ELISA plates. Antigens were coated in 96-wells ELISA plates (Maxisorb, NUNC) in 50 mM NaHCO\textsubscript{3}, pH 9.6, in 50 µl per well. After overnight coating at 4°C, wells were washed with PBS and blocked with 5% non-fat dried milk powder in PBS (MPBS). Wells were incubated with 100 µl phages (approximately 1x10\textsuperscript{10} cfu/ml), diluted with 100 µl MPBS containing 0.05% Tween-20 (MPBST), during 90 minutes at 37°C. In case of GST fusion proteins (hPop1 and Rpp38) as antigen, 10 µg soluble GST was

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Oligonucleotides used for library construction</th>
</tr>
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<tbody>
<tr>
<td>CSCVHo-F</td>
<td>5′ GGTCAGTCCTCTAGATCTTCCGCCGTGACGTTGGACGAG 3′</td>
</tr>
<tr>
<td>CSCVHo-FL</td>
<td>5′ GGTCAGTCCTCTAGATCTTCCGGCGGTGGTGGCAGCTCCGGTGGTGGCGGTTCCGCCCTGACGTTGGACGAG 3′</td>
</tr>
<tr>
<td>CSCG-B</td>
<td>5′ CTGGCCGGCCTGGCCACTAGTGGAGGAGACGATGACTTCGGTCC 3′</td>
</tr>
<tr>
<td>CSCK-V</td>
<td>5′ GTGGCCCAGGCGGCCCTGACTCAGCCGTCCTCGGTGTC 3′</td>
</tr>
<tr>
<td>CKJo-B</td>
<td>5′ GGAAGATCTAGAGGACTGACCTAGGACGGTCAGG 3′</td>
</tr>
<tr>
<td>CSC-F</td>
<td>5′ GAGGAGGAGGAGGAGGAGGTCGCCCAGGCGGCCCTGACTCAG 3′</td>
</tr>
<tr>
<td>CSC-B</td>
<td>5′ GAGGAGGAGGAGGAGGAGGAGCTGGCCGGCCTGGCCACTAGTGGAGG 3′</td>
</tr>
<tr>
<td>CSCVK</td>
<td>5′ GTGGCCCAGGCGGCCCTGACTCAGCCGTCCTCGGTGTC 3′</td>
</tr>
<tr>
<td>CKJo-B</td>
<td>5′ GGAAGATCTAGAGGACTGACCTAGGACGGTCAGG 3′</td>
</tr>
</tbody>
</table>
added to the phage solution during selection. After panning, wells were washed several times with PBS containing 0.05% Tween-20 (PBST). Bound phages were eluted with 100 mM triethylamine or 100 mM glycine-HCl, pH 2, and neutralized with Tris-HCl, pH 7.4, prior to infection of E.coli TG1. After several selection rounds, polyclonal phage populations were analyzed in ELISA for reactivity with the antigens, as described below.

In a first experiment, all antigens except Rrp4 and Rrp42 were coated, at a concentration of 4 µg per well for the first selection round, and at 0.4 µg per well for following selection rounds. After five selection rounds, polyclonal phage ELISA demonstrated that the phage pools were not detectably enriched for phages directed to hPop1, HspB3, Topo, Ro60, and Rrp30. In a second experiment, these antigens were coated at a concentration of 0.5 µg per well in all selection rounds. In a third experiment, phages were selected on Rrp4, Rrp42, and again on annexin XI, α-fodrin, hPop1, Ro52, Rpp38, and U1A. The antigens were coated at a concentration of 0.5 µg per well in the first selection round, and at a concentration of 0.25 µg per well in next selection rounds.

During single colony analysis, monoclonal phages were examined for binding activity in ELISA as described below, and the scFv gene was analyzed for full cDNA insert by PCR using primers pCOMB-F2548 (TTCCGGCTCGTATGTTGTGTG) and pCOMB-R3010 (GAATCAAGTTTGCCTTTAGCGTC) were sequenced using primers pComb-F2548 (TTCCGGCTCGTATGTTGTGTG) and pComb-R3010 (GAATCAAGTTTGCCTTTAGCGTC).

**ELISA**

Antigens were coated in 96-wells ELISA plates (Maxisorb, NUNC) in 50 mM NaHCO₃, pH 9.6, in 50 µl per well, overnight at 4°C. Antigens were coated at a concentration of 0.25 µg per well, except Rrp4 and Rrp42, which were coated at a concentration of 0.125 µg per well. Plates were blocked with MPBS for one hour. Subsequently, plates were incubated for one hour with the phage solutions, diluted in MPBPI. In case of GST fusion proteins, competing soluble GST was added at a ten-fold excess. To detect bound phages, plates were washed with PBST and incubated with mouse anti-M13 monoclonal antibody (GE Healthcare), 1,000-fold diluted in MPBPI, followed by incubation with horse radish peroxidase (HRP)-labelled rabbit anti-mouse antibodies (DAKO), 1,000-fold diluted in MPBPI. Plates were washed with PBST and PBS. Bound HRP-conjugated antibodies were detected by conversion of 3,3’,5,5’-tetramethylbenzidine (TMB). Reactions were stopped with 1 M H₂SO₄, and the absorbance at 450 nm was measured. Pre-immune and immune serum were analyzed in ELISA as well. Bound chicken antibodies were detected using HRP-labelled rabbit anti-chicken antibodies (Jackson ImmunoResearch), 1,500-fold diluted in MPBPI, followed by TMB reaction as described above.

**Cell culture**

HeLa cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) medium supplemented with 10% foetal calf serum, 1 mM penicillin and 1 mM streptomycin. The cells were grown in a humidified incubator at 37°C in the presence of 5% CO₂. HeLa cells were scraped in PBS, harvested by centrifugation at 8,000g for 5 minutes and washed twice with PBS. Cell extract was prepared by sonication in sonification buffer (50 mM Tris-HCl pH 7.6, 10 mM KCl, 0.05% Nonidet-P40, 1 mM EDTA, and 1 mM dithiothreitol), followed by centrifugation at 12,000g for 20 minutes. The clear lysate was stored at −70°C.

**Immunoblot analysis**

Proteins from HeLa cell extract were separated on polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes by semi-dry electrophotting. All immunoblotting steps were carried out at room temperature on a shaking table. The membrane was blocked with MPBST. Phages were incubated with the membrane at a concentration of approximately 5x10⁴ cfu/ml in MPBPI. After extensive washing with MPBPI, the membrane was incubated with HRP-labelled mouse anti-M13 monoclonal antibody (GE Healthcare), 5,000-fold diluted. Finally, bound HRP-labelled antibodies were visualized using enhanced chemiluminescence (ECL).

**References**


Recombinant antibody expression vectors enabling double- and triple-immunostaining of tissue culture cells using monoclonal antibodies

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Double- and triple- immunostaining

Next to the already available mouse monoclonal and laboratory animal-derived polyclonal antibodies, recombinant antibodies offer an additional and virtually unlimited arsenal of new immunohistochemical research tools. The major advantages of recombinant antibodies are their rapid and easy generation against virtually any target. The avidity of antibody fragments can be increased by partial dimerisation. This can be achieved by fusion of $C_L$ domains derived of different species to recombinant antibody domains. The $V_L$-linker-$V_H$-$C_L$ constructs result in significantly lower dimerisation levels compared to the $V_H$-linker-$V_L$-$C_L$ antibody constructs. The most efficient dimerisation occurs with the Jun-tagged scFvs. The very large and rapidly expanding collection of recombinant antibodies already available combined with the ease of introducing various tag sequences allows for an almost unrestricted number of easily adjustable research tools. To our best knowledge we report for the first time that using $C_L$ domains derived from different species, in combination with readily available commercial secondary antibodies specific for these $C_L$ domains, provides an easy method for the application of recombinant monoclonal antibodies of various origins in immunohistochemical analyses eliminating the problem of co-staining with multiple mono- or polyclonal antibodies. Both double and triple labelling experiments can be performed successfully.

Introduction

The rapidly expanding repertoire of recombinant antibodies generated from various sources provides new powerful monoclonal antibody tools for immunological analysis of cells and tissues. In order to enable easy double- and triple-label immunostaining of cells and tissues with sets of such monoclonal antibodies we devised a set of vectors containing expression cassettes for the expression of recombinant antibodies as a fusion protein with the immunoglobulin light chain constant domain ($C_L$) of either mouse, chicken or human origin (see Figure 1). Recombinant antibodies expressed using these vectors, in combination with readily available commercial secondary antibodies conjugated to different fluorophores, form a powerful source of tools that allow the easy setup of double or triple labelling immunostaining assays.

Acknowledgements

This research was in part funded by a Bio-partner First Stage Grant, and ModiQuest B.V. Nijmegen, The Netherlands. We thank Zheng Qui for her assistance in the western blotting experiments.
Results and discussion

Dimerisation behaviour and relative expression levels of the various subtypes of recombinant antibodies were analyzed using denaturing and non-denaturing western blots of the recombinant antibodies derived from different expression vectors and different recombinant antibody systems. We have compared recombinant human antibodies (V_H-linker-V_L-tag) with recombinant chicken (V_L-linker-V_H-tag) derived clones. In general we observed that the expression levels of the chicken V_L-linker-V_H-tag clones were lower compared to the human V_H-linker-V_L-tag clones. Furthermore, the C_L-domains in combination with the human recombinant antibodies resulted in a higher percentage of dimerisation compared to the chicken scFvs fused to identical tags. Only the Jun dimerisation domains resulted in efficient dimerisation under the conditions tested. Low levels of proteolytic cleavage were observed in all C_L-tag scFv antibodies, but seemed to be more pronounced in the chicken derived scFv clones (Figure 2).

Double and triple labelling of HeLa and HEp-2 cells was easily achieved after initial titration of the concentrations of recombinant and mouse monoclonal antibodies. The individual antibodies for the double labelling experiments were chosen for their differential staining of nucleolar (Rpp30 protein (1)), and cytoplasmic cytoskeletal proteins (i.e. vimentin (2) and cytokeratin). The antibodies for the triple labelling stained nucleolar (fibrillarin protein (3)), nuclear (U1C protein (4)), and cytoplasmic (Ribo-P proteins (5)) proteins. Initially, in a sequential incubation procedure, every individual antibody was incubated with...
the fixed cells, followed by a wash step, after which the next antibody was added. This time-consuming method was successful, albeit the staining intensities of some recombinant antibodies were weaker compared to single-label immunostaining experiments using the respective antibodies. The observed decrease in staining intensity is probably due to the relative long incubation times required for the sequential staining procedure. Eventually, all labelling experiments were performed as a two-step method. All primary antibodies were mixed and incubated with the fixed cells followed by a wash step. Subsequently, all secondary antibodies were mixed and incubated with the cells, again followed by a wash step. Cells were embedded in mowiol, and directly analysed with either an epifluorescence microscope (Figure 3a) or a confocal microscope (Figure 3b). Both fixation methods used gave similar staining patterns.

Summary

Next to the already available mouse monoclonal and laboratory animal-derived polyclonal antibodies, recombinant antibodies offer an additional and virtually unlimited arsenal of new immunohistochemical research tools. The major advantages of recombinant antibodies are their rapid and easy generation against virtually any target. The avidity of antibody fragments can be increased by partial dimerisation. This can be achieved by fusion of Cκ domains derived of different species to recombinant antibody domains. The V\textsubscript{L}-linker-\textsubscript{Cκ} constructs result in significantly lower dimerisation levels compared to the V\textsubscript{L}-linker-\textsubscript{C\textsubscript{λ}} antibody constructs. The most efficient dimerisation occurs with the Jun-tagged scFvs. The very large and rapidly expanding collection of recombinant antibodies already available combined with the ease of introducing various tag sequences allows for an almost unrestricted number of easily adjustable research tools. To our best knowledge we report for the first time that using C\textsubscript{λ} domains derived from different species, in combination with readily available commercial secondary antibodies specific for these C\textsubscript{λ} domains, provides an easy method for the application of recombinant monoclonal antibodies of various origins in immunohistochemical analyses eliminating the problem of co-staining with multiple mono- or polyclonal antibodies. Both double and triple labelling experiments can be performed successfully.

### Table 1. Oligonucleotides used for assembly of scFv expression vectors

| C\textsubscript{mm}ForNot-I | 5’GAAGATCTCGCGGCTGCACACTCATTCCTGTTGAAGCTC3’ |
| C\textsubscript{mm}BackBgl-II | 5’GAGTCATTCTCGACTTGCGGCCGCTCAGCCCAAGGTGGCCCCCACCATC3’ |
| C\textsubscript{ch}ForNot-I | 5’GAAGATCTCGCGGCTGCACACTCTCCCCTGTTGAAGCTCTTTGTGAC3’ |
| C\textsubscript{ch}BackBgl-II | 5’GAAGATCTCGCGGCTGCACACTCATTCCTGTTGAAGCTC3’ |

### Materials and Methods

#### Introduction of C\textsubscript{λ}-domain-tags in recombinant antibody expression vectors

The mouse C\textsubscript{λ}-domain, the human C\textsubscript{λ}-domain, and the chicken C\textsubscript{λ}-domain were amplified from; cDNA derived from mouse spleen lymphocytes using oligos CmForNot-I and CmBackBgl-II (6), from the vector pCOMb3-TT (7) using oligos C\textsubscript{λ}chForNot-I and C\textsubscript{λ}chBackBgl-II, and from cDNA derived from chicken spleen lymphocytes using oligos C\textsubscript{λ}chForNot-I and C\textsubscript{λ}chBackBgl-II, respectively. The resulting PCR fragments were digested with Not-I and Bgl-II and cloned in a Not-I and Bgl-II digested pUFosVH6 vector (6) resulting in pUCm\textsubscript{κ}-VH6, pUCch\textsubscript{κ}-VH6, and pUCch\textsubscript{λ}-VH6, respectively (Figure 1). All oligonucleotides used are listed in Table 1.

#### Monoclonal antibodies

Recombinant monoclonal antibodies from various sources were cloned in the different expression vectors. The Ra2 human recombinant scFv antibody isolated from a human rheumatoid arthritis patient derived phage display library (8) recognising a flaggrin-derived peptide sequence and cytoskeletal intermediate filaments keratin 8 and vimentin, was cloned in pUCch\textsubscript{λ}-VH6. The recombinant anti-human Rpp30 (a component of the nucleolar RNase-MRP and RNase-P complexes) (1) chicken antibody, isolated from an immunized chicken-derived phage display library (Raats, unpublished data) was cloned in pUCm\textsubscript{κ}-VH6. The anti-U1C human recombinant antibody K36 isolated from a human systemic lupus erythematosus (SLE) patient derived phage display library (9) was cloned in pUCch\textsubscript{λ}-VH6. The anti-Ribosomal P proteins antibody A4 isolated from a human SLE patient derived phage display library (10) was cloned in the pUCch\textsubscript{λ}-VH6. Finally, the mouse monoclonal anti-human fibrillarin 72B9 (a component of the nucleolar hU3-snORNP complex) was used in its original complete IgG2a format (11). Recombinant antibodies were used at concentrations of 0.5 mg/ml. Tissue culture supernatant of mouse monoclonal antibody 72B9 was used at a dilution of 1:10.

#### Western blot analysis of expressed recombinant antibodies

All recombinant antibody clones were expressed as periplasmic fractions as described previously (12). ScFv were induced by 1 mM IPTG for 3 hours at 37°C, whereas C\textsubscript{λ}-domain scFvs and Jun dimers (6) were induced by 1 mM IPTG for 15 hours at 20°C. Recombinant antibodies were analysed on 10% polyacrylamide denaturing and non-denaturing SDS-PAGE, and semi-dry blotted onto nitrocellulose membranes. Blots were blocked for one hour in MPBST (5% non-fat milk, 0.05% Tween 20, in PBS). Antibodies were visualised using anti-V5- or anti-Flag-antibodies (Dako, Glostrup, Denmark) and HRP-labelled rabbit anti-mouse antibodies (Dako, Glostrup, Denmark), 2,500-fold diluted. Bound antibodies were detected by enhanced chemiluminescence (ECL).

#### Indirect immunofluorescence staining

ScFvs were purified from periplasmic fractions by immobilised metal chelate affinity chromatography (IMAC), and dialysed overnight against PBS pH 8.0 as described previously (13). HeLa and Hep-2 cells were cultured in DMEM ( Dulbecco’s modified Eagle’s medium) supplemented with 10% foetal calf serum, 1 mM penicillin and 1 mM streptomycin. For the indirect immunofluorescence staining procedure, HeLa and Hep-2 cells were grown on...
cover slips, and fixed in methanol/acetic acid (14) or in p-formaldehyde (15). The cells were incubated with mixtures of the monoclonal antibodies (mAb) (1:1:1 working dilutions; 0.5 mg/ml recombinant mAb or 1:10 mouse mAb) for 1.5 hours, followed by three quick washes with PBS and detection with a mixture of the appropriate fluorochrome labelled secondary antibodies, sheep anti-mouse-Texas Red (Amersham Biosciences Europe GmbH, Roosendaal, Netherlands), rabbit anti-chicken-FITC (Jackson Laboratories Inc. West Grove, USA), sheep anti-mouse-Texas Red (Amersham Biosciences Europe GmbH, Roosendaal, Netherlands), and rabbit anti-chicken-FITC (Jackson Laboratories Inc. West Grove, USA), sheep anti-mouse-Texas Red (Amersham Biosciences Europe GmbH, Roosendaal, Netherlands), in a dilution of 1:100, 1:50 and 1:50, respectively (see Figure 3a). Cells were embedded in Mowiol (16) and analysed using an epifluorescence microscope or a BioRad MRC 1000 confocal laser scanning microscope.

References

Chapter 4

A novel subtractive antibody phage display method to discover disease markers

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Today’s research demands fast identification of potential diagnostic and therapeutic targets. We describe a novel phage display strategy to identify disease-related proteins that are specifically expressed in a certain (diseased) tissue or cells. Phages displaying antibody fragments are selected on complex protein mixtures in a two-step manner, combining subtractive selection in solution with further enrichment of specific phages on two-dimensional western blots. Targets recognized by the resulting recombinant antibodies are immunoaffinity purified and identified by mass spectrometry. We used antibody fragment libraries from autoimmune patients to discover apoptosis-specific and disease-related targets. One of the three identified targets is the U1-70K protein, a marker for SLE overlap disease. Interestingly, the epitope on U1-70K, recognized by the selected recombinant antibody, is shown to be apoptosis-dependent, and such epitopes are believed to be involved in breaking tolerance to self-antigens. The other two proteins are identified as PSF/p54nrb and hnRNP C.

Introduction

In the post-genomics era, where the human genome is completely sequenced and available in databases, it became clear that having the complete genome sequence is not enough to reveal biological function. For example, mRNA levels do not necessarily correlate with the expression levels of the corresponding proteins, and the relative levels of protein variants resulting from either alternative splicing or posttranslational modifications cannot be predicted from the gene sequence. ‘Proteomics’ defines a broad area of interest and is aimed at protein identification, studying protein function, posttranslational modifications, protein-protein interactions and finding molecular drug targets (1). Such molecular drug targets can be proteins that are specifically present in a diseased tissue or cell. One approach to discover ‘proteome-specific’ proteins is to compare the proteome of a diseased cell or tissue with the proteome of the same cell or tissue in a normal (non-diseased) state. This is commonly done by two-dimensional gel electrophoresis and mass spectrometry. Such an approach generally leads to an (extensive) list of proteins that are up- or down-regulated or modified in various ways. To subsequently find the proteins that are actually relevant for disease is a difficult and laborious task. Phage display, first described by George Smith in 1985 (2), is a powerful technique based on the display of proteins or peptides on the surface of phage particles that carry the corresponding gene. In proteomics, phage display offers an elegant approach in the search for disease markers. Subtractive antibody selections have been successfully performed on whole cells (3-10), 

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tissue sections (11), biomaterials ex vivo (12), and cell extracts (13-19). A major advantage of subtractive phage display technology is the simultaneous generation of recombinant monoclonal antibodies recognizing potential disease markers. Such antigen-antibody pairs could be directly applicable in therapeutics or diagnostics.

Here, we present a novel subtractive antibody phage display method that enables the identification of proteome-specific, intra-cellular epitopes. As schematically represented in Figure 1, the selection method comprises two steps. In the first step, phages are selected in solution on the proteome of interest with prior subtraction on a comparable ‘normal’ proteome (Figure 1a and 1b). Biotinylation of the proteomes allows their immobilization on magnetic streptavidin-coated beads. In the second phase, specific phages from the phage pool, enriched for phages to proteome-specific epitopes in the first step, are further selected on a two-dimensional western blot of the proteome of interest (Figure 1c). After identification of proteome-specific phages, their targets are immunoaffinity purified using these recombinant antibodies, and identified by means of mass spectrometry. The use of immune libraries (for example libraries from autoimmune patients) will enhance the chances of discovering disease-related epitopes, since these libraries will contain high affinity antibodies against disease markers. In systemic autoimmune diseases, it still remains poorly understood why patients produce antibodies to self-antigens. One (more and more supported) theory points at the involvement of aberrant protein modifications, for example those occurring during apoptosis. Such modifications are believed to reveal ‘cryptic’ epitopes on autoantigens and as such play a role in breaking tolerance to self-antigens (20-22). In the current study, the subtractive phage display selection method was applied, using autoimmune patient antibody libraries, to identify apoptosis-specific epitopes relevant for systemic autoimmune diseases. Three epitopes were identified that are exclusively present in the apoptotic cell extract. One of these epitopes is associated with the U1-70K protein, which is cleaved during apoptosis and which is an important marker for systemic lupus erythematosus (SLE) overlap disease (also named mixed connective tissue disease, MCTD). Finally, we demonstrate that the recognition of this epitope by the selected antibody is dependent on the apoptotic modification.

![Figure 1. Schematic representation of the selection strategy.](image)

The selection strategy is a combination of several subtractive selection rounds on proteomes in solution, followed by an enrichment step on immunoblot. Phages are selected on biotinylated proteomes, with subsequent capture by magnetic streptavidin-coated beads. Phages recognizing common epitopes are first removed by a subtractive selection on the comparable ‘normal’ proteome (a), and subsequently remaining phages are selected on epitopes that are specifically present in the proteome of interest (b). These selection rounds are repeated several times. Subsequently, western blots containing both proteomes are incubated with the selected phage pool, enriched for antibodies to proteome-specific epitopes (c). Spots are excised from the membrane, bound phages are eluted and used to infect bacterial cultures to isolate monoclonal phages. Finally, these phages are screened for recognition of proteome-specific epitopes.

### Results

**Test selections on biotinylated cytoplasmic HeLa cell extracts**

Successful selections against complex mixtures of antigens, such as a cellular extract, depend on many factors (23), including the amount and concentration of antigen, and the affinity of the antibody fragments. Selection conditions were tested by means of mock selections. A semi-synthetic antibody phage library was simultaneously spiked with phages displaying monoclonal antibody fragments recognizing the autoantigens La and Ro52, previously selected from SLE derived libraries ((124), unpublished results), at various ratios. The 1:10<sup>6</sup> ratio approximated the complexity of the libraries used in subsequent experiments. After selection on biotinylated cytoplasmic HeLa cell extract, the polyclonal phage pools were tested in enzyme-linked immunosorbent assay (ELISA) for reactivity against La and Ro52, and against bovine serum albumin (BSA) as a control antigen. As shown in Figure 2, at a 1:10<sup>6</sup> ratio anti-La phages were detectably enriched after just one selection round, whereas at a 1:10<sup>7</sup> ratio anti-La phages were detected after two rounds of panning. At a 1:10<sup>6</sup> ratio the polyclonal phage stock showed slight reactivity with Ro52, whereas at a 1:10<sup>7</sup> ratio no reactivity against Ro52 was observed after two selection rounds. After three selection rounds, 96 monoclonal phages were analyzed for reactivity in ELISA, and 27% of the phages reacted with La, whereas 10% was positive.
Eight hours after induction with anisomycin, the cleavage of the U1-70K protein to a 450 nm was measured.

Figure 2. Test selections of polyclonal phages using biotinylated cytoplasmic HeLa cell extract. In the test selections, anti-La and anti-Ro52 phages were simultaneously added to a semi-synthetic library at a 1:10 ratio (a) and at a 1:100 ratio (b), and selected on 1.5 µg biotinylated cytoplasmic HeLa cell extract, captured using magnetic streptavidin-coated beads. The phage populations resulting from the selection rounds were tested for binding to La and Ro52. BSA was used as a negative control. Selection rounds are numbered (0, 1, and 2). Per well 0.1 µg recombinant protein was coated. Polyclonal phage pools were diluted to a concentration of 10^5 cfu/ml and detected using HRP-labelled anti-M13 mAb. The ELISA plates were developed with TMB as a substrate, and after the addition of H_2SO_4, the absorption at 450 nm was measured.

Subtractive selection of recombinant antibodies against apoptosis-specific epitopes

To induce apoptosis, HeLa and Jurkat cells were incubated with anisomycin for eight hours. As a biochemical marker for apoptosis the cleavage of the U1-70K protein to a 40 kDa apoptosis-specific fragment, an early event during apoptosis (26), was monitored. Eight hours after induction with anisomycin, the apoptotic 40 kDa fragment was detected by immunoblotting, whereas the full-length 70 kDa protein was reduced to undetectable levels (data not shown), indicating that the cells had entered the apoptotic pathway.

Libraries of rheumatoid arthritis (RA), systemic sclerosis (SSc) and SLE patients (24,27,28) were mixed using equal amounts of phages from each library, and phages were selected for reactivity with apoptosis-specific epitopes by subtractive selection as detailed in the Materials and Methods section. After three selection rounds, phage populations of each selection round were analyzed on small two-dimensional western blots containing non-apoptotic and apoptotic cell extracts. The polyclonal phage stock obtained after the third selection round showed the strongest reactivity on western blot, and was chosen for further analysis (Figure 3). Several of the apoptosis-specific spots were excised and bound phages were eluted. Both enhanced chemiluminescence (ECL) detection and 3,3',5,5'-tetramethylbenzidine (TMB) staining were examined as detection methods. Where TMB staining is more accurate (due to a direct precipitate on the membrane), ECL detection resulted in a higher sensitivity. The amount of phages eluted from ECL-detected spots was approximately equal to the amount of phages eluted from TMB-stained spots, and no negative effect on phage infectivity was observed for both staining methods. In average, 100 phages eluted per spot. Phages were selected, based on full-length cDNA insert and expression of soluble single chain variable fragments (scFv). Fingerprint analysis of phages with full-length cDNA and good scFv expression resulted in a number of groups, and randomly, one clone per group was analyzed for reactivity on immunoblot containing both extracts. All scFvs were derived from their original spots.

Approximately 40 µg of non-apoptotic cytoplasmic HeLa cell extract (a) and apoptotic HeLa cell extract (b) was separated by two-dimensional electrophoresis. In the first dimension, proteins were separated by isoelectric focusing on immobilized pH gradients with a pH range from 3 to 10, and in the second dimension by SDS-PAGE on a 12.5% polyacrylamide gel. Molecular weights of marker proteins (kDa) are indicated on the left. After two-dimensional electrophoresis, proteins were transferred to a nitrocellulose membrane and the membrane was blocked with MBBST. Prior to the immunoblot analysis, phages were selected during three consecutive subtractive selection rounds on biotinylated apoptotic cell extract. Phages were incubated with the pre-blocked membrane at a concentration of 5x10^7 cfu/ml and detected with HRP-labelled anti-M13 mAb and ECL. Between antibody incubations, the membrane was washed extensively with MBBST to reduce background on the film. A similar (TMB-stained) membrane was used for excision of apoptosis-specific spots.
Figure 4. Characterization of the reactivities of the selected monoclonal recombinant antibodies. (a-c) Immunoblotting. Apoptosis was induced in Jurkat cells by incubation with anisomycin for eight hours. Cell extracts were prepared by sonication. Approximately 10 µg of apoptotic (a) and non-apoptotic (n) Jurkat cell extract was separated by SDS-PAGE on a 12.5% polyacrylamide gel and electroblotted onto nitrocellulose membranes. Molecular weights of marker proteins (kDa) are indicated on the left. ScFvs were isolated from the periplasm. ScFvs B1H11 and R3A4 were incubated with the membrane at a 10-fold dilution, and scFv G1G10 at a 20-fold dilution. ScFv B1H11 (a) recognizes protein bands of 100, 55 and 45 kDa in non-apoptotic cell extract, and additional strong protein bands of 50 and 42 kDa in apoptotic cell extract. Another additional protein band, running slightly faster than the 100 kDa band, is recognized relatively weaker. ScFv G1G10 (b) reacts with a protein band of approximately 38 kDa in non-apoptotic cell extract and with a protein band of approximately 37 kDa in apoptotic cell extract. ScFv R3A4 (c) recognizes a protein band of 70 kDa in non-apoptotic cell extract, and a 40 kDa protein band in apoptotic cell extract. Note that the reactivity with the apoptotic form of this protein is stronger than the reactivity with the non-apoptotic form. (e-g) Immunofluorescence analysis. HeLa monolayer cells were fixed and incubated with purified scFvs. Bound scFvs were detected by anti-6xHis-mAb and FITC-labelled rabbit anti-mouse antibodies. ScFv B1H11 (e), G1G10 (f) and R3A4 (g) all give a nucleoplasmic staining. (d) Immunoaffinity purification. ScFvs G1G10 (lane 1) and B1H11 (lane 2) were isolated during the selection procedure, can serve as tools to identify and characterize the corresponding target antigens. ScFvs were C-terminally tagged with a 6xHis-tag, immobilized on nickel beads, and target proteins were then isolated from cell extracts and separated on SDS-PAGE gels. As shown in Figure 4d, scFv B1H11 efficiently immunoprecipitated proteins with molecular weights corresponding to those detected by immunoblotting, whereas immunooaffinity purification of the target recognized by scFv G1G10 appeared to be less efficient. These protein bands were excised from the gel and the proteins in the gel slices identified by mass spectrometry. An overview of the selected targets and their characteristics is presented in Table 1.

The proteins recognized by scFv B1H11 were identified as polypyrimidine-tract binding protein (PTB)-associated splicing factor (PSF, 54 kDa) and p54 nuclear RNA binding protein (p54nrb, 54 kDa). The scFv recognizes both PSF and p54nrb on immunoblot, indicating that the epitope recognized by this scFv is present on both proteins. In agreement with this, the two proteins are approximately 70% identical on the level of amino acids over a region of 328 residues, strongly suggesting that the epitope is located in this region of these molecules. Interestingly, it has been reported that PSF is hyperphosphorylated (29) and p54nrb is cleaved (30) during apoptosis. Cleavage of p54nrb leads to a size reduction of approximately 4 kDa, which agrees well with the apoptosis-specific protein band of 50 kDa (Figure 4a). The 45 kDa protein band in non-apoptotic cell extract is presumably either a splicing variant or degradation product of p54nrb. Also this variant seems to be cleaved in apoptotic cells leading to the appearance of the 42 kDa species, which is also recognized by scFv B1H11. In agreement with the nuclear localization of PSF and p54nrb, immunofluorescence analysis of scFv B1H11 demonstrated a nucleoplasmic staining (Figure 4e).

The protein recognized by scFv G1G10 was identified by mass spectrometric analysis as heterogeneous nuclear ribonucleoprotein C (hnRNP C). As a result of alternative splicing, two variants of hnRNP C can be detected in most cells, designated C1 and C2, which differ approximately 3 kDa in size (31). The identity of the G1G10 target protein was confirmed by the reactivity of scFv G1G10 with hnRNP C, immunoprecipitated from Jurkat cells using an anti-hnRNP C-specific patient serum (32). During apoptosis, hnRNP C1 and C2 are known to be cleaved, resulting in a size reduction of approximately 2 kDa (33), which is consistent with the finding that the protein recognized by scFv G1G10 in apoptotic cell extract migrates slightly faster in SDS-PAGE than the antigen present in non-apoptotic cell extract (Figure 4b). ScFv G1G10 resulted in a nucleoplasmic staining (Figure 4f), consistent with the localization of hnRNP C (33).

Immunoblot analysis showed that scFv R3A4 recognized a protein band of 70 kDa in non-apoptotic cell extract, and a 40 kDa protein band in apoptotic cell extract (Figure 4c), which is reminiscent of the U1-70K protein and its apoptotic cleavage product (26). The U1-70K protein is a well-known autoantigen in SLE overlap syndrome and recognition...
of the cleavage product by autoantibodies (21) suggested that scFv R3A4, which was isolated from a patient-derived antibody library, might be targeting the U1-70K protein. A number of experiments demonstrated that this indeed was the case. First, the scFv R3A4 reacted with recombinant U1-70K (a truncation mutant consisting of the first 195 amino acids of U1-70K) in ELISA (data not shown). Second, immunofluorescence analysis, as shown in Figure 4g, revealed that the cellular localization of the target antigen was nucleoplasmic (speckled pattern), corresponding to the localization of the U1-70K protein. Finally, the sequence of scFv R3A4 cDNA appeared to be almost identical to the V\_\_ and V\_\_ sequences of scFvs directed to the U1-70K protein, which were previously selected from SLE libraries in our laboratory (34).

**Identification of an apoptosis-dependent epitope on the U1-70K protein**

Interestingly, scFv R3A4 appeared to react stronger with apoptotic than with non-apoptotic U1-70K on immunoblots (Figure 4c). Similar observations were previously reported by Degen et al. for other anti-U1-70K scFvs (34). We hypothesized that the efficiency by which the epitope is recognized by these scFvs is dependent on the apoptotic modification. To study the in vitro reactivity of anti-U1-70K antibodies with apoptotic and non-apoptotic U1-70K, anti-U1-70K antibodies (i.e. scFv R3A4, three scFvs previously selected from SLE libraries and designated scFv-4, -6 and -7 (34), and mouse monoclonal antibody 2.73 (35)) were used to immunoprecipitate U1 small nuclear ribonucleoprotein (U1 snRNP) particles from apoptotic and non-apoptotic cell extracts. The U1 snRNP complex consists of the U1 snRNA molecule, three U1-specific proteins (U1A, U1C and U1-70K) and a ring of seven distinct Sm proteins. The 40 kDa apoptotic fragment of the U1-70K protein remains specifically present on the apoptotic fragment. Mouse monoclonal antibody 2.73 reacted much stronger with apoptotic U1-70K than with apoptotic U1-70K, which agrees with its preferential recognition of the full-length U1-70K in immunoblotting (21).

**Discussion**

Here, we describe a novel antibody phage display method to identify proteome-specific epitopes that are relevant for disease. Recombinant autoantibodies from patient libraries were selected on apoptotic cell extract,

![Figure 5. U1 snRNP immunoprecipitation by anti-U1-70K scFvs.](image-url)
after subtraction on non-apoptotic cell extract, to identify disease-related targets that are specifically present in the apoptotic material. All target antigens isolated as a result of this selection strategy are proteins that are modified during apoptosis. Moreover, two of the three targets are well-documented autoantigens, with the U1-70K protein being an important marker for SLE overlap disease. HnRNP C is autoantigenic as well, although autoantibodies to this protein have been reported in a few patients only (32,33). So far, PSF and p54\textsuperscript{rf} have not been described as autoantigens, but one of the patients from the libraries was found to possess (low titres of) antibodies to recombinant p54\textsuperscript{rf} by immunoblotting. The autoantigenic U1-70K protein is of particular interest, since it was recently demonstrated that this protein contains an apoptosis-specific B-cell epitope, which is believed to be involved in breaking immunological tolerance to the U1-70K protein (20,21,37). We demonstrated that the anti-U1-70K scFv R3A4 reacts in \textit{vitro} with an apoptosis-specific epitope on U1-70K as well. The recognition of intact U1-70K on immunoblots is believed to result from unfolding of the protein due to the electrophoresis procedure.

Using large naïve libraries, the selection strategy presented here is generally applicable for the discovery of proteome-specific epitopes, as for example proteins that are exclusively present on tumour cells, or proteins that are specifically expressed during different life stages of the malaria parasite. The technique is especially suitable for the discovery of markers that are related to diseases in which the immune system is involved, such as autoimmune diseases. Since libraries of such patients contain high-affinity antibodies, the use of these libraries will bias towards the selection of (the most important) disease-related epitopes.

So far, selections on complex mixtures of intracellular antigens have been mainly performed using immobilization on immunotubes (13,15) or blotting membranes (16,18,19). The advantage of biotinylation (in combination with magnetic streptavidin-coated beads) is that panning on antigens can be performed in solution, resulting in an optimal accessibility of the epitopes for the phages (38). The test selections, performed with anti-Ro52 and anti-La spiked libraries, demonstrated that phages from autoimmune libraries can be selected using small amounts of biotinylated cell extracts, which would allow simultaneous competition with a large excess of the non-biotinylated ‘normal’ proteome. It is possible that epitopes are changed due to the attachment of biotin groups to the protein, but this potential problem was minimized by choosing a relatively low biotin:protein ratio. Another advantage of our approach is the combinatorial approach of selection in solution and selection on two-dimensional western blots, which decreases the amount of individual monoclonal phages to be analyzed. To our knowledge, we are the first to report such a combinatorial approach.

In the current study, phages were selected on proteins in solution during three subtractive selection rounds prior to selection on immunoblots. It is generally accepted that in such procedures diversity decreases with the progression of the selection procedure. If a higher diversity is preferred, one might continue with the phages that are selected during the first or second selection rounds. On the other hand, a less diverse phage population (such as obtained after three or more selection rounds) might give a better representation of the important targets in the antigen mixtures. Moreover, after the first and second selection rounds, the concentrations of individual phages might be too low for detection of the antigens on two-dimensional immunoblots, the second selection step in the procedure described here.

Individual phages, selected on two-dimensional immunoblots, were screened on one-dimensional immunoblots to validate their reactivities. We then focused on three targets that were differentially detected in apoptotic cell extracts compared to non-apoptotic cell extracts. Obviously, an automated high-throughput screening system would greatly facilitate the simultaneous isolation of more possible targets. Besides, by screening on immunoblots of SDS-PAGE gels, the screening was now restricted to differences between proteomes with regard to molecular weight. Combining this screening with other assays, such as immunoblots of one-dimensional isoelectric focusing gels, a capture ELISA, or a filter lift assay (39), would also augment the number of possible disease markers.

In general, the affinity of scFvs for antigen-binding is lower compared to complete immunoglobulins, and immunoaffinity purification may therefore be less efficient. So far, a few studies have successfully used scFvs to isolate the unknown target antigen and to determine its identity by mass spectrometry (5,6). We demonstrate that scFvs, isolated during the subtractive selection strategy presented here, can be applied successfully to immunoaffinity purify target proteins for identification by mass spectrometry. A critical parameter is the amount of cell extract needed. However, the development of more sensitive mass spectrometry equipment will allow the identification of target proteins with smaller amounts of protein, and thus with less cell extract.

The post-genomics era demands innovative methods for fast target identification for a number of applications. The method described here offers an attractive platform for the discovery of potential therapeutic and diagnostic targets, especially in combination with a high-throughput screening system.

Materials and methods

Phage display libraries and patient sera

Patients were classified in accordance with standard criteria for each autoimmune disease. Autoimmune patient libraries of SLE (patients D3, D8, D18, D18t, O11 and Z2), SSC (patients B92, H248, J70, S185 and T3) and RA (patients Du, He and Wy) patients were available in pHenIX-VSV vector format (24,27,28). The synthetic library made by Nissim (40) was used for test selections. To reduce the background of phages that do not express a functional antibody-gene III fusion protein, and to reduce the number of selection rounds needed, a trypsin sensitive helper phage (41) was used for phage amplification. Serum samples were provided by patients seen at the Department of Rheumatology and Internal Medicine of the Radboud University Nijmegen Medical Centre (The Netherlands).
Cell lines, induction of cell death and preparation of cell extracts

Jurkat (human T cell leukemia) suspension cells were grown in RPMI 1640 medium (Gibco-BRL), supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin and 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL), in a humidified 37°C incubator containing 5% CO2. Jurkat cells were maintained at a concentration of 1x10^6 cells/ml. HeLa cells were either purchased from Computer Cell Culture Centre (Mons, Belgium), or HeLa suspension cells were grown in S-MEM medium with Joklik modification (Oxoid), supplemented with non-essential amino acids (Gibco-BRL), 5% newborn serum (NBS, Gibco-BRL), 2 mM L-glutamine (Gibco-BRL), 1 mM penicillin and 1 mM streptomycin. Apoptosis was induced in Jurkat and HeLa cells by addition of 10 µg/ml anisomycin. Eight hours after induction, cells were harvested by centrifugation at 800g for 5 minutes and washed with PBS. Prior to the washing procedure, HeLa cells attached to the culture flask were scraped in PBS and pooled with the HeLa cells that are released from the flask into the culture medium due to the apoptotic process. Non-apoptotic HeLa cytoplasmic extract was prepared as described (42). Cell extracts were prepared by three different methods: i) cells were resuspended in PBS and lysed by freezing-thawing, ii) cells were resuspended in lysis buffer (50 mM Tris-HCl pH 7.6, 100 mM KCl, 0.05% Nonidet-P-40 (NP40), 1 mM EDTA, and 1 mM dithioerythritol (DTE)) and lysed by sonicating, or iii) cells were resuspended in NP40 lysis buffer (25 mM Tris-HCl pH 7.6, 100 mM KCl, 1% NP40, 10 mM MgCl2, 0.25 mM DTE) and lysed on ice for 30 minutes. Cell lysates were prepared, at a concentration of 0.5 – 1 x 10^6 cells/ml, on ice, and in the presence of protease inhibitors (Complete™ protease inhibitor cocktail (Roche)). After cell lysis, extracts were centrifuged at 12,000g and 4°C for 30 minutes, and clear lysates were stored at −70°C. Three mg cell extract (soluble proteins) was biotinylated with 150 µg NHS-LC-biotin (Pierce) in 50 mM NaHCO3 pH 8.3, and dialyzed against 0.5 mM DTE and 20% glycerol in PBS. The protein concentration of the biotinylated cell extract was measured using a bicinchoninic acid (BCA) protein assay (Pierce) with bovine serum albumin BSA as a standard, and the amount of magnetic streptavidin beads (DYNAL, Biotech) needed to completely capture a given amount of biotinylated cell extract was established empirically.

Protein electrophoresis and immunoblotting

Proteins were separated by one- or two-dimensional electrophoresis. For isoelectric focusing immobilized pH gradient strips were used (Pharmacia). Proteins were stained with colloidal Coomassie (SERTA) or by silver staining. Alternatively, proteins were transferred to a nitrocellulose (Schleicher&Schuell) or polyvinylidene fluoride (PVDF; Millipore) membrane by electroblotting. For immunoblotting, all incubation steps were carried out at room temperature on a shaking table. Blocking solutions consisted of PBS, 0.05% Tween-20, and either 5% non-fat dried milk powder, or 1% gelatine. Gelatine was only applied when phages were used for detection. ScFv were isolated from biotinylated mouse monoclonal antibody (GE Healthcare), followed by TMB (NEOGEN) staining (44) or ECL.

Phage selection

Test selections on biotinylated HeLa cytoplasmic cell extract

To test whether selection against biotinylated cell extract was promising, anti-La and anti-Ro52 phages, previously selected from human autoimmune patient derived libraries ((24), unpublished results), were diluted in a semi- synthetic (Nissim) phagemid library at two different ratios, namely a 1:10^5 ratio and a 1:10^6 ratio. For a 1:10^5 ratio 10^3 anti-La and 10^2 anti-Ro52 phages were simultaneously mixed with 10^6 phages of the semi-synthetic library, and for a 1:10^6 ratio 10^4 anti-La and 10^5 anti-Ro52 were simultaneously mixed with 10^7 phages of the semi-synthetic library. All panning steps were performed using an end-over-end rotator at room temperature. First, the phages were incubated with 1 mg magnetic streptavidin beads, pre-blocked with 1.5 µg biotinylated normal HeLa cytoplasmic extract for one hour, 1 mg pre-blocked magnetic streptavidin beads were added and the mixture was rotated for 15 minutes. Beads were magnetically separated from the solution and discarded, while the solution containing the remaining phages was transferred to a clean tube. Subsequently, phages were incubated with 1.5 µg biotinylated normal HeLa cytoplasmic cell extract for one hour, 1 mg pre-blocked magnetic streptavidin beads were added and the mixture was rotated for 15 minutes. Beads were magnetically harvested from the solution and washed several times with PBS containing 0.05% Tween-20 (PBST) and twice with PBS. Bound phages were eluted by incubating the washed beads in 1 ml trypsin (10 g/l in PBS, pH 7.4 and pre-warmed at 37°C) for 30 minutes at room temperature. Beads were harvested and discarded, whereas the solution containing the eluted phages was transferred to a clean tube and incubated with 1 ml of NBS for 5 minutes to inhibit trypsin activity. Phages were amplified using trypsin sensitive helper phage and purified from the medium by polyethylene glycol (PEG) precipitation as described (45).

Polyclonal phage ELISA

Polyclonal phage pools of each round were screened in ELISA for reactivity with La and Ro52, and against BSA as a control. Antigens were coated on 96 wells maxisorb microtitre plates (NUNC) at a concentration of 0.1 µg per well in 100 µl 50 mM NaHCO3 pH 9.3, overnight at 4°C. Plates were blocked with 400 µl MPBS, containing 0.05% Tween-20 (MPBST) per well at room temperature for two hours. Phages were diluted in MPBST at a concentration of 10^5 cfu/ml, and 100 µl was added at room temperature for one hour. The plates were washed eight times with PBST, and subsequently HRP-conjugated anti-M13 monoclonal antibody (mAb) (Pharmacia) was added at a 5,000-fold dilution in MPBST at room temperature for one hour. Plates were washed eight times with PBST and twice with PBS. Bound HRP-conjugated antibodies were detected by TMB conversion. Reactions were stopped with 1 M H2SO4 and the absorbance at 450 nm was measured.

Subtractive selection against biotinylated apoptotic cell extract

Libraries of RA, SLE and SS patients were equally mixed and used for selection. All panning steps were performed using an end-over-end rotator at room temperature. First, the phages were incubated with 1 mg magnetic streptavidin beads, pre-blocked with
 Target discovery by antibody phage display

5% non-fat dried milk powder and 1% BSA in PBS, for one hour. Beads were magnetically separated from the solution and discarded, while the solution containing the remaining phages was transferred to a clean tube. To subtract phages recognizing normal cell extract components, phages were incubated with 1.5 µg biotinylated non-apoptotic HeLa cytoplasmic cell extract for one hour, 1 mg pre-blocked magnetic streptavidin beads were added and the mixture was rotated for 15 minutes. Beads were harvested and discarded, and the remaining phage solution was transferred to a clean tube. This subtraction step was repeated with another 1.5 µg of biotinylated non-apoptotic HeLa cytoplasmic cell extract and 1 mg pre-blocked beads. Remaining phages were again transferred to a clean tube. Subsequently, phages specifically recognizing apoptotic cell extract components were selected by panning against 1.5 µg biotinylated apoptotic HeLa cell extract for one hour. 1 mg pre-blocked magnetic streptavidin beads were added and the mixture was rotated for 15 minutes. Beads were harvested, washed several times with PBS and, bound phages were eluted by incubating the beads in 1 ml trypsin (10 mg/ml in PBS, pH 7.4) for 30 minutes. Beads were discarded and eluted phages were incubated with 1 ml NBS, and immediately used to infect exponentially growing E.coli. Phages were then amplified and purified as described above.

**Selection of enriched phage populations on 2D western blots**

After three subtractive selection rounds on biotinylated apoptotic cell extract as described above, polyclonal phage pools of each round were analyzed on western blots of two-dimensional gels containing apoptotic and non-apoptotic cell extracts, as described above. The phage stock from the third round showed highest signals and most differences between apoptotic and non-apoptotic extracts. To specifically select phages recognizing a protein spot that was differentially recognized in apoptotic versus non-apoptotic cell extracts, western blot membranes of two-dimensional gels were incubated with the phage stock from the third selection round as described above. Directly after visualization of bound phages, the membrane was covered with PBS and membrane spots of interest were accurately excised, using a clean razor blade for each spot. TMB staining resulted in a blue precipitate on the membrane, allowing highly accurate excision of spots. For ECL detection, the film was placed on a light box and exactly overlaid with the membrane. Bound phages were eluted from the excised spots by incubating the blot piece in 100 mM triethylamine (TEA), transferred to a clean tube and neutralized with 1 M Tris-HCl pH 7.4. The eluted phages were subsequently treated with trypsin, as described above, to reduce background. Finally, phages were used to infect exponentially growing E.coli/TG1 and plated on square (12 cm x 12 cm) 2xTY agar plates containing 100 µg/ml ampicillin and 2% glucose.

**Screening of single colonies**

Single colonies were amplified for VSV-G-expression by a dot blot assay, and for full length DNA by PCR, using primers LMR3 (CAGGACAGCTATGACCATG) and FdSeq (GTAACGATCTAAAGTTTTGTCG). Full-length clones with good expression levels were fingerprinted with the restriction enzyme BstNI. From all unique fingerprint clone prints with good expression, phages were produced in 2 ml-deepwell microtiter plates. Undiluted culture supernatants, containing phage, were screened on western blots of SDS-PAGE gels containing non-apoptotic and apoptotic cell extracts. Phages were detected with HRP-labelled anti-M13 monoclonal antibody as described above. Several phages recognized proteins that were differentially present between non-apoptotic and apoptotic cell extracts. Three clones were chosen for further analysis, and their cDNAs were sequenced as described (46).

**Immunoaffinity purification and identification of target antigens**

ScFvs were C-terminally tagged with a 6xHis-tag by cloning the cDNA in a pUC19 vector (45) via compatible Ncol and NotI digestion. Recombinant scFv antibody fragments were isolated from 80 ml E.coli periplasm as previously described (43) and dialyzed against p-IPP500 buffer (50 mM phosphate buffer, pH 8.0, 500 mM NaCl). All steps were performed at 4°C using an end-over-end rotator. The scFv solution was incubated with 50 µl Ni-NTA agarose beads (50% slurry, Qiagen) for 2 hours. Subsequently, beads were washed three times with 15 ml IPP500 and three times with 15 ml p-IPP150 (50 mM phosphate buffer, pH 8.0, 150 mM NaCl). Beads were incubated with cell extract of 5x10⁵ cells, prepared by sonication, at a concentration of 5x10⁵ cells/ml and membrane spots of interest were accurately bound phages, the membrane was covered with PBS and immediately used to infect exponentially growing E.coli/TG1. Phages were then amplified and purified as described above.

**U1 snRNP immunoprecipitation**

All incubations were performed in t-IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP40) at 4°C using an end-over-end rotator. Between incubations, beads were washed three times with t-IPP150. Protein A-agarose beads (20 µl of 50% slurry, Kem-En-Tec) were pre-coated with 12.5 µl rabbit anti-mouse IgG (Dako) for 2 hours and subsequently coated with 50 µl mouse anti-VSV-G mAb for 2 hours. Then, beads were incubated with 5 ml concentrated solution of VSV-G-tagged recombinant scFv antibody fragments, isolated from E.coli periplasm as described above. Finally, beads were incubated with 60 µl of 5x10⁶ cells/ml Jurkat apoptotic or non-apoptotic cell extract, prepared by lysis in NP40 lysis buffer as described above, for 2 hours. Mouse mAb 2.73, directed against U1-70K protein was used as a positive control. Anti-BSA scFv, previously selected.
from an SLE patient library (unpublished results), was used as a negative control. Finally, beads were resuspended in 100 µl t-IPP150, containing 0.5% SDS, and co-immunoprecipitated RNA was isolated by addition of TRIzol reagent (Gibco-BRL) according to the manufacturer’s protocol. Isolated RNA was subsequently size-fractionated on 6% polyacrylamide/8M urea gels and transferred to a nylon membrane (HybondTM-N, GE Healthcare) by northern blotting. Finally, U1 RNA was detected using a 32P-labelled U1 RNA specific anti-sense probe and analyzed by autoradiography. The signals were quantified using a ScanMaker 8700 (MICROTEK). The U1 snRNA was isolated from the apoptotic and non-apoptotic Jurkat cells as well, and size-fractionated on the same gel (10% of the input material).

References


Part 2

Autoantigenicity of cell death-associated epitopes
Chapter 5

Introduction to cell death-specific epitopes on autoantigens: Apoptotic modifications affect the autoreactivity of the U1 snRNP autoantigen

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A hallmark of systemic autoimmune diseases is the presence of high titers of serum autoantibodies targeting a diversity of autoantigens. Most components of the U1 snRNP complex are autoantigenic in systemic lupus erythematosus and systemic lupus erythematosus overlap syndrome, which is also called mixed connective tissue disease. It is hypothesized that posttranslational modifications, in particular cell death-specific modifications, play an important role in breaking tolerance to self-antigens. Recently, it became clear that the U1 snRNP particle, more specifically its U1-70K protein component, displays a new epitope during apoptosis. This chapter intends to give an overview of the modifications that occur on the U1 snRNP autoantigens, especially those arising during cell death, as an introduction to cell death-specific epitopes on autoantigens.

The U1 snRNP particle

Pre-mRNA splicing, the removal of non-coding introns from mRNA precursors to obtain functional mRNA, which then serves as a template for protein synthesis, is one of the many essential processes in the eukaryotic cell. The main player in splicing is the spliceosome, a complex macromolecular machinery that consists of several uridine-rich small nuclear ribonucleoproteins (U snRNPs), and many non-RNP splicing factors (1). An important constituent of the spliceosome is the U1 snRNP particle, which is the first of the U snRNPs to bind to the pre-mRNA (1). It associates with the 5’-splice site through base pairing of the single-stranded 5’ terminal sequence element of the U1 snRNA molecule with conserved residues at the 5’ splice site of the pre-mRNA, which subsequently leads to functional pairing of the intronic 5’ and 3’ splice sites (2). Additionally, the U1 snRNP has been reported to be involved in the regulation of poly(A) tail synthesis (3). The U1 snRNP complex consists of the 165 nucleotide U1 snRNA molecule, the U1-specific proteins U1A, U1C, and U1-70K, and a doughnut-shaped structure consisting of seven proteins, called Sm proteins (B/B’, D1, D2, D3, E, F and G), which are core components contained in all U snRNPs (U1, U2, U4/U6 and U5) (4). Recently, the structure of the U1 snRNP complex has been determined by cryo-electron microscopy (2). It is believed that the seven relatively small Sm proteins form a core with the snRNA, and that the larger U1 snRNP-specific proteins determine the specific structure of the snRNP particle.

The U1 snRNP autoantigen

Originally, autoreactivity in patient sera against the U1 snRNP particle was termed anti-RNP. This reactivity was found to be related though not identical to anti-Sm reactivity (5). The elucidation of the molecular composition of the U snRNPs clarified that anti-RNP sera contained autoantibodies targeting at least

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one of the U1 snRNP-specific proteins U1A, U1C and U1-70K, whereas anti-Sm sera are directed to at least one of the Sm proteins and as a consequence are directed to the other U snRNPs in addition to the U1 snRNP (6). Anti-RNP sera may also contain autoantibodies directed to the RNA component of this particle, the U1 snRNA (7). Later, it was demonstrated that some patient sera contain autoreactivity to both the U1 and the U2 snRNPs, but not to the other U snRNPs. These autoantibodies appeared to be directed to epitopes shared by the structurally related U1A and U2B’ proteins (8).

Autoantibodies targeting the U1A, U1C and U1-70K proteins and the U1 snRNA molecule are mainly found in patients suffering from systemic lupus erythematosus (SLE) overlap syndrome, whereas autoantibodies directed to Sm-D, Sm-B/B’, and the E-F-G complex are more specifically associated with SLE (9,10).

**Hypothesis of cell death-specific epitopes involved in breaking tolerance to self-antigens**

The autoimmune response is a complex process, of which the underlying molecular mechanisms are still only poorly understood, and in which (a combination of) genetic, hormonal and environmental factors are believed to be involved. For the autoimmune response to Sm-B/B’ the molecular mimicry model, in which it is hypothesized that for example a viral infection can elicit an immune response to self-antigens through cross-reactivity with a viral epitope, is favored (11,12). This model is illustrated by the fact that the Sm-B/B’ protein contains a three-fold repeat of a proline rich sequence element, which constitutes a major Sm epitope in anti-Sm sera, and which is similar to a proline rich sequence found in the Epstein-Barr virus nuclear antigen 1 (EBNA-1). Another model, which is increasingly supported by experimental data obtained during the last decade, hypothesizes as mentioned before that uncommon posttranslational modifications play an important role in breaking tolerance to (certain) self-antigens (11,13,14). In this context, dying cells represent an interesting source of modified antigens. During apoptosis and necrosis many known autoantigens are modified, as for example by proteolytic cleavage, by hyper- or dephosphorylation, and by citrullination (11,13-15). Also oxidative fragmentation of proteins has been put forward as a cause of autoantigen modification (16,17). It is believed that such modified autoantigens reveal novel ‘cryptic’ epitopes that trigger the primary immune response, which is subsequently followed by the generation of autoreactivity to other, non-modification-dependent epitopes by epitope spreading. In this process, the secondary immune responses may spread intramolecularily, but also intermolecularily to stably associated proteins (18). The recent data on the immune responses to the U1 snRNP particle and in particular the studies performed on the U1-70K protein, which will be discussed here, strongly support this theory. Figure 1 depicts the U1 snRNP particle and summarizes the modifications of its individual components.

**Different isoforms of U1 snRNP components**

The molecular diversity of the products from a single gene can be generated in different ways. A major mechanism leading to the synthesis of different isoforms in higher eukaryotes is alternative splicing of pre-mRNAs. Another important process that increases the molecular heterogeneity of products from a single gene is posttranslational modification. The relative expression levels of different protein isoforms may differ between different cell types and/or tissues and may also be affected by the state of the cell (e.g. phase of the cell cycle). Most of the current knowledge on the different isoforms of U1 snRNP components has been obtained by the analysis of material from cultured cell lines.

**Isoforms of the U1-70K protein**

Database analysis revealed that at least four alternative splice variants of the U1-70K protein exist in humans. Isoforms 1 and 2 differ only by the presence or absence of 9 amino acids in the centre of the sequence (aa 223-231 in isoform 1) as a result of an alternative splice site. Experimental evidence to support the physiological relevance of isoform 3 (containing the N-terminal 159 amino acids of isoform 1 and 7 other amino acids at its C-terminus) and isoform 4 (in which the N-terminal 159 amino acids of isoform 1 are replaced by 63 other amino acids) is still very poor.

In 1990, Woppmann et al. analyzed human snRNP particles isolated from HeLa cells by two-dimensional gel electrophoresis and observed at least thirteen U1-70K isoforms in a pi range of 6.7 to 8.8 (19). It was shown that the U1-70K variants have four major phosphopeptides in common, suggesting that U1-70K is constitutively phosphorylated at four serine residues. Additionally, they found three other serine residues that were differentially phosphorylated in the various U1-70K isoforms. However, phosphorylation alone could not explain the existence of so many isoforms, and it was proposed that other (yet unidentified) posttranslational modifications are present in U1-70K as well (19). Recently, Monneaux et al. demonstrated the importance for antigenicity of phosphorylation on a specific serine residue of U1-70K in MRL/lpr mice (lupus-prone mice) (20). Previously these investigators identified an epitope at residues 131 – 150 of the U1-70K protein that was recognized by antibodies as well as CD4+ T cells in two strains of lupus mice (21). They subsequently synthesized two peptides, one with a phosphate group on serine residue 140 and one with a non-phosphorylated peptide, whereas 12 of the 15 lupus patients recognized the peptide with Ser140 phosphate group on serine residue 140. It was demonstrated that the peptide with Ser140, but not the peptide with Ser140, was recognized by antibodies and CD4+ T cells from MRL/lpr mice. Interestingly, the Ser140 peptide, when administered intravenously in saline solution, appeared to significantly decrease both proteinuria and the production of anti-DNA antibodies in MRL/lpr mice. Furthermore, 9 out of 15 lupus patients reacted with the non-phosphorylated peptide, whereas 12 of the same 15 lupus patients recognized the peptide with phosphorylated Ser140 (20), indicating that this phosphorylation site is important for autoantibody recognition as well, and might play a role in the autoimmune response to U1-70K.
Isoforms of the U1A and U1C protein

Except for the removal of the N-terminal methionine and subsequent acetylation of the N-terminus, no heterogeneity has been reported for the U1A protein. Also the U1C protein does not seem to be expressed in various isoforms, although there are some indications that this protein may be posttranslationally modified based upon its electrophoretic behavior in SDS-PAGE gels (22).

Isoforms of the Sm proteins

Sm-B and Sm-B’ are produced from the same gene by alternative splicing and as a result they contain slightly different sequences at their C-termini. The Sm-D1, -D2 and -D3 proteins are each encoded by a distinct gene, although their sequences do share similarities. It has been demonstrated that the C-terminal arginine-glycine (RG) repeats of the human Sm-D1, Sm-D2 and Sm-B/B’ contain symmetric dimethylarginines (sDMAs) in normal cells (23). The sDMA-modified C-terminus of Sm-D1 was shown to constitute a major linear epitope for anti-Sm autoantibodies, which suggested that also non-apoptotic posttranslational modifications of Sm proteins may play a role in the aetiology of autoantibody formation in SLE (23). For the relatively small Sm proteins, E, F and G, so far no variants have been reported.

Cell death-associated modifications of the U1 snRNP

Apoptotic modifications of U1 snRNP components have been studied extensively, and modifications occurring during cell death have been identified and characterized for the U1 snRNA molecule, and for the U1-70K and Sm-F proteins. Additionally, hyperphosphorylated splicing factors associate with the U1 snRNP particle during apoptosis. To date, no apoptotic or necrotic modifications of the autoantigenic proteins U1A, U1C, and the other Sm proteins have been reported.

Apoptotic cleavage of U1 snRNA

The U1 snRNA molecule is modified during apoptosis by the specific removal of 5 or 6 nucleotides from the 5’ end, which includes the 2,2,7-trimethylguanosine cap (24). Recently, Hoffman et al. revealed that the U1 snRNA molecule was able to stimulate the toll-like receptor 3 (TLR-3) at similar efficiencies as the known TLR-3 agonist poly(IC) (25). This results in the secretion of type 1 interferons, and these molecules have been associated with the development of autoimmune disease. The results of this study suggest that the U1 snRNA molecule itself is capable to elicit an immune response (25). Interestingly, anti-U1 snRNA autoantibody levels have been described to correlate with disease activity in patients suffering from SLE overlap syndrome and are believed to have predictive value for disease flares (26). It is currently unknown whether the apoptotic cleavage close to the 5’ end of the U1 snRNA affects its antigenicity.

Cleavage of the U1-70K protein during cell death

Since Casciola-Rosen et al. demonstrated in 1994 that the specific cleavage of the U1-70K protein early during apoptosis is a biochemical hallmark of apoptosis (27), U1-70K cleavage is often used as a marker for apoptosis. The U1-70K protein was found to be cleaved at the C-terminal side of D341-DGPD341 by caspase-3, resulting in an N-terminal 40 kDa (aa 1-341) and a C-terminal 22 kDa (aa 342-437) protein fragment (28). Degen et al. demonstrated that the U1-70K protein is cleaved while it is associated with the intact U1 snRNP particle in apoptotic cells, and that the N-terminal 40 kDa fragment remains associated with the U1 snRNP complex (24). In addition, Casciola-Rosen et al. observed that during apoptosis, autoantigens become clustered in two distinct populations of blebs at the cell surface (29). Using affinity-purified anti-U1-70K antibodies, it was revealed that U1-70K was localized in the nucleus early in the apoptotic process (stained as a rim around the condensed nucleus), and that U1-70K epitopes accumulated in the apoptotic bodies in the later phases of apoptosis (29). It is
thought that the translocation of autoantigens to the fragile apoptotic vesicles enhances the chance that they are exposed to the immune system.

The fate of the U1-70K protein during other forms of cell death has been studied as well. The U1-70K protein appeared to be degraded into untraceable fragments during primary (mercury-induced) necrosis (30) and secondary necrosis (prolonged exposure to an apoptosis-inducing agent such as etoposide) (15). During CTL-induced apoptosis, the U1-70K protein is cleaved by granzyme B into a 60 kDa fragment (31). Furthermore, the U1-70K protein can be fragmented by metal-catalyzed oxidation, which generates several fragments between 33 and 38 kDa (16). Interestingly, Greidinger et al. showed that the preferential recognition of apoptotically and oxidatively modified forms of the U1-70K autoantigen was associated with distinct clinical rheumatic disease manifestations (17). SLE patients with lupus skin disease showed increased reactivity with caspase-cleaved U1-70K, whereas sera from SLE patients with Raynaud’s phenomenon preferentially reacted with oxidatively modified U1-70K.

**Cleavage of the Sm-F protein during apoptosis**

Anti-Sm autoantibodies are almost exclusively found in SLE patients (4), and the fate of the Sm proteins during cell death has therefore been studied extensively. The only modification occurring on the Sm proteins during cell death that has been identified so far is the apoptotic cleavage of the Sm-F protein (32), a component of the autoantigenic E-F-G complex. Cleavage of the 11 kDa Sm-F protein results in a 9 kDa apoptotic fragment that remains associated with the U snRNP complexes in apoptotic cells (32). This finding suggests a possible role for the apoptotic modification of the Sm-F protein in triggering the autoimmune response against the Sm proteins, and consequently anti-Sm autoantibody formation in patients.

**Association of phosphorylated splicing factors with the U1 snRNP during apoptosis**

In 1998, Utz et al. noticed that phosphorylated serine/arginine (SR) splicing factors specifically associated with the U1 snRNP particle during apoptosis (33). It was shown that monospecific sera and monoclonal antibodies recognizing the U1 snRNP complex specifically immunoprecipitated a phosphoprotein complex from apoptotic ^3P-labelled Jurkat cell lysates. The phosphoprotein complex appeared to consist of the four proteins pp54, pp42, pp34, and pp23. In 2000, Neugebauer et al. analyzed a cohort of autoimmune patients by ELISA for reactivity with phosphorylated SR proteins, purified from HeLa cells, and showed that SR proteins are targets of autoantibodies in approximately 50% of patients suffering from SLE (34). Interestingly, they showed that sera containing high reactivities against SR proteins were only weakly reactive with dsDNA, and vice versa. Furthermore, it was tested whether phosphorylation directly influenced the recognition of the SR proteins by autoantibodies. Remarkably, some patient sera showed decreased reactivity with dephosphorylated SR proteins, whereas other patient sera displayed the opposite behaviour (34).

**The immune response to the U1 snRNP**

In 2001, it was shown that the appearance of autoantibodies to components of the U1 snRNP particle follows a characteristic order, with U1-70K and Sm-B/B’ as important early immunogens (35). At the start of this study, a large cohort of patients was analyzed, on clinical grounds, for the presence of autoantibodies to RNP proteins. The presence of such antibodies was detected using immunoblots of Jurkat lysates containing the U1 polypeptides. Within this panel, 109 patients did not have detectable levels of autoantibodies directed to any of the U1 snRNP polypeptides in the first serum sample. However, these patients developed such anti-RNP autoantibodies in time (this patient group was defined as ‘monospecific seroconverters’). An additional group of 54 patients was selected, consisting of patients that initially tested negative for autoantibodies to at least one of the RNP polypeptides, and subsequently developed autoantibodies to this polypeptide (so-called ‘late seroconverters’). Subsequently, serum serum samples of these patients were tested for the presence and first appearance of autoantibodies to the individual U1 polypeptides on immunoblot. It was observed that autoantibodies to the U1-70K and Sm-B/B’ proteins more frequently appeared early in the anti-RNP response than antibodies to other U1 snRNP polypeptides. Moreover, it was demonstrated that autoantibodies directed to U1A and U1C in general developed after the appearance of other anti-RNP autoantibodies, and that autoantibodies targeting Sm-D were detected only after the appearance of several autoantibodies to other U1 snRNP polypeptides. Autoantibodies directed to the U1 snRNA molecule were not included in this study. The finding that U1-70K is a major early immunogen, taken together with the fact that U1-70K is modified during apoptosis, is consistent with the hypothesis that apoptotic modifications on U1-70K protein might be important for triggering of the immune response to the U1 snRNP particle (35).

**An apoptosis-specific epitope on the U1-70K protein**

In 2000, Degen et al. isolated a panel of monoclonal recombinant autoantibodies directed to U1-70K from phage display libraries derived from SLE patients. Interestingly, several of these single chain variable fragments preferentially recognized apoptotic U1-70K (i.e. the 40 kDa fragment) on immunoblot (36). This not only indicated that the apoptotically cleaved U1-70K polypeptide contains an epitope that is not exposed by the full-length protein, but also suggested that autoimmune patients may produce antibodies that specifically target this epitope. The existence of such autoantibodies in patient sera was substantiated by the observation that a subgroup of SLE patients displays increased reactivity with caspase-cleaved U1-70K in comparison with oxidatively fragmented U1-70K (17). More recently, Greidinger et al. confirmed the presence of autoantibodies to caspase-cleaved U1-70K by screening a cohort of anti-RNP positive sera by immunoblotting for the occurrence of autoantibodies recognizing apoptotic U1-70K in the presence of...
soluble U1-70K to block other autoantibodies to this protein. It was shown that 15 of 29 sera with antibodies directed to U1-70K contained apoptosis-specific anti-U1-70K antibodies. In addition, antibodies to apoptotic U1-70K were found in 2 of 25 sera without detectable levels of anti-U1-70K antibodies (37). This finding was recently confirmed by a study performed in our laboratory. We analyzed a cohort of patients suffering from SLE overlap syndrome, SLE and non-SLE autoimmune diseases (such as rheumatoid arthritis or systemic sclerosis) for the presence of antibodies to apoptotic and intact U1-70K protein by immunoblotting. We showed that the majority of patients suffering from SLE overlap syndrome (54%) had higher reactivities to apoptotic U1-70K than to intact U1-70K, whereas in only a few patients intact U1-70K was more efficiently recognized than apoptotic U1-70K (38). In the same study, a longitudinal analysis of twelve anti-RNP positive patients demonstrated that several early sera are relatively enriched for autoantibodies recognizing an apoptosis-specific epitope on U1-70K (38), supporting the hypothesis that apoptotic U1-70K is involved in the triggering of the primary immune response to the U1-70K protein and the U1 snRNP particle. Immediately, it has been hypothesized that novel ‘cryptic’ epitopes might be created due to posttranslational modifications, but only recently such an epitope has been characterized for the first time. Greidinger et al. demonstrated that the apoptotic U1-70K protein displays a B-cell epitope that is not found on the intact protein, the formation of which is dependent on residues 180 – 205 (39).

In this study, seven patient sera were used that recognized apoptotic U1-70K much more efficiently than the intact form. These sera were analyzed for reactivity with recombinant, C-terminally truncated U1-70K mutants on immunoblot and in ELISA. All seven sera strongly recognized the apoptotic form consisting of aa 1 – 341 (the caspase-3 cleavage site is positioned between aa 341 and 342), as well as C-terminally truncated forms down to aa 205. Five of the seven sera did not recognize a further C-terminally truncated form consisting of aa 1 – 180, and none of the seven sera recognized a C-terminally truncated form of aa 1 – 150. None of the sera reacted with a synthetic peptide comprising aa 180 – 205 in ELISA. Additionally, this 25-mer was neither able to block recognition of the 1 – 205 mutant in ELISA, nor to block recognition of apoptotic U1-70K in immunoblotting. The apoptosis-specific epitope is therefore believed to be dependent on conformational constraints, and it is thought that the presence of other residues than those between 180 and 205 contributes to the formation of the epitope (39). Interestingly, the apoptosis-specific epitope on the U1-70K protein appeared to overlap partially with the apoptosis-specific epitope on the U1-70K (38), supporting the hypothesis that several early sera are relatively enriched for the presence of antibodies to apoptotic and intact U1-70K protein by immunoblotting. The apoptosis-specific epitope on the U1-70K protein appeared to overlap partially with the apoptosis-specific epitope on the U1-70K (38), supporting the hypothesis that several early sera are relatively enriched for the presence of antibodies to apoptotic and intact U1-70K protein by immunoblotting.

Concluding remarks

As described above and summarized in Figure 1, a number of posttranslational and cell death-associated modifications occur on components of the U1 snRNP particle. One of the remaining questions is why, upon exposure of apoptotically modified U1 snRNP to the immune system, some patients develop antibodies to Sm-F (and the E-F-G complex), whereas other patients generate an immune response to U1-70K (and subsequently U1A and U1C).

References


Autoantibodies specific for apoptotic U1-70K are superior serological markers for mixed connective tissue disease

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Modifications occurring on autoantigens during cell death have been hypothesized to play a role in the initiation of autoimmune diseases. Patients suffering from mixed connective tissue disease produce autoantibodies directed to U1 snRNP and antibodies against a 70 kDa protein component, the U1-70K protein, are the most prominent. During apoptosis, U1-70K is cleaved by caspase-3 to a 40 kDa product, which remains associated with the complex. Autoantibodies preferentially recognizing the apoptotic form of U1-70K have been described previously, and an apoptosis-specific epitope on U1-70K has been identified. This study shows that 29 of 53 (54%) mixed connective tissue disease sera preferentially recognize the apoptotic form of U1-70K over intact U1-70K. Moreover, we show that antibodies directed to an apoptosis-specific epitope on U1-70K are more specifically associated with MCTD than other anti-U1-70K antibodies, suggesting that apoptotic U1-70K is a better antigen for the detection of these antibodies in MCTD patients. Longitudinal analysis of twelve MCTD patients demonstrated in several patients that early sera are relatively enriched with antibodies recognizing an apoptosis-specific epitope, and that the levels of these apoptosis-specific antibodies decrease in time. These findings indicate that the early detection of apoptotic U1-70K is of considerable interest for anti-U1 snRNP positive patients.

Introduction

Patients suffering from autoimmune diseases are characterized by the presence of autoantibodies directed to a wide range of autoantigens. Mixed connective tissue disease (MCTD) is a relatively rare systemic autoimmune disease, and includes a group of patients with overlapping clinical symptoms of systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA) and polymyositis/dermatomyositis (PM/DM). Sharp et al. were the first to describe MCTD as a distinct rheumatic disease (1), but whether MCTD can be regarded as a distinct disorder has been subject of discussion (2).

A characteristic serological feature that distinguishes MCTD patients from patients with other connective tissue diseases are high levels of autoantibodies directed against the U1 small nuclear ribonucleoprotein (snRNP) particle (1,3). The U1 snRNP is a highly conserved RNA-protein complex, located in the nucleus, where it is involved in the processing of pre-mRNA (4,5). It consists of the U1 snRNA molecule and several proteins: the U1A, U1C and U1-70K proteins are components specific for the U1 snRNP, whereas the eight Sm proteins (B/B’, D1, D2, D3, E, F and G) are shared with other U snRNPs (6). Most

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U1 snRNP components are autoantigenic in MCTD and SLE. Autoantibodies directed against U1A, U1C, U1-70K and the U1 snRNP molecule are mainly found in MCTD patients, whereas autoantibodies targeting Sm-D, Sm-B/B’ and the E-F-G complex are more specifically associated with SLE (7,8).

The mechanisms through which such auto-antigens, generally highly conserved and ubiquitously expressed molecules, escape tolerance and are recognized by the immune system as non-self remain unclear, but it is hypothesized that cell death plays an important role in the initiation of autoimmune responses (9,10). Recently, also secondary necrosis has been put forward as a source of proteolytically modified autoantigens (11), but the modifications that occur on autoantigens during apoptosis were studied most extensively. Apoptotic modifications on autoantigens include specific cleavage by caspas or granzyme B, (hyper)phosphorylation, dephosphorylation, citrullination, methylation and trans-glutaminase cross-linking (10,12,13), and it is thought that these modifications might be seen by the immune system as novel ‘cryptic’ epitopes. It is believed that these novel epitopes induce the primary immune response, and that secondary immune responses and epitope spreading result in autoantibodies that are directed against unmodified regions of the autoantigens and antigens that are associated with the initially modified autoantigen (9).

One of the apoptotic modifications occurring on the U1 snRNP is cleavage of U1-70K at residue 341 by caspase-3 (14,15). Antibodies against U1-70K are in general the first autoantibodies to appear in anti-U1 snRNP (often referred to as anti-RNP) positive patients, indicating that U1-70K is important as an initial autoantigen (16). The molecular and immunological characteristics of the major apoptotic isoform of U1-70K, a 40 kDa cleavage product which remains associated with the U1 snRNP complex (17), and its role in the triggering of the primary and possibly secondary autoimmune response, are therefore intriguing.

Recently it was shown that sera of some anti-U1 snRNP positive patients contain antibodies that specifically bind to the apoptotic form of U1-70K, which displays an epitope that is not present on the intact form (18,19). This epitope is dependent on the region between amino acids 180 and 205, partially overlapping with the RNA binding domain and overlapping with the most common T-cell epitope (20).

In this study we analyzed a cohort of MCTD and control patients for the presence of autoantibodies against intact and apoptotic U1-70K. Moreover, we longitudinally analyzed sera from another group of MCTD patients. Our results show that early in disease, autoantibodies directed against the apoptotic form of U1-70K (70Kapop) are more strongly represented than autoantibodies against the intact form. Longitudinal studies also show that autoantibodies against 70Kapop do not significantly correlate with disease flares.

Results

In this study, patient sera were analyzed for the presence of autoantibodies against U1-70K (70K) and its apoptotic product (70Kapop), on western blots containing extracts of non-apoptotic and apoptotic Jurkat cells. Two positive controls for the detection of 70K and 70Kapop were included in each experiment: anti-U1-70K mouse monoclonal antibody 2.73 (21), which displays higher reactivities with 70K than with 70Kapop, and patient serum B16, which reacts with both 70K and 70Kapop. The position of 70Kapop on western blots was confirmed by a recombinant monoclonal antibody, recognizing both 70K and 70Kapop (Figure 1a) (22). The results demonstrate that in these apoptotic cells U1-70K is almost completely converted into 70Kapop. Besides positive controls for 70K and 70Kapop, mouse monoclonal antibody ANA125 directed against Sm-B/B’ (Figure 1a), and anti-U1A/ U2-B’ mouse monoclonal antibody 9A9 (not shown) were used as well. To be able to detect autoantibody reactivities to the intact U1-70K and its apoptotic 40 kDa fragment simultaneously and to facilitate a direct comparison of these reactivities, a mixture of apoptotic and non-apoptotic cell extracts was used to prepare western blots. An additional advantage of this approach was that inter-blot differences could be excluded, thereby allowing a more accurate comparison of reactivities with 70K and 70Kapop in a single patient serum. Serum antibody reactivities against 70K and 70Kapop were scored ranging from 0 to 5. Figure 1b shows a western blot containing such a mixture of non-apoptotic and apoptotic Jurkat cell extracts, probed with a serial dilution of MCTD patient serum B16. It is shown that the signals for 70K and 70Kapop increase when the serum is applied at a lower dilution, indicating that the western blot assay can be used for semi-quantitative interpretation.

Autoantibodies against U1-70K are more easily detected using apoptotic U1-70K

The presence of high levels of autoantibodies directed against components of the U1 snRNP, such as 70K, is one of the criteria for the diagnosis of MCTD (2). However, anti-70K antibodies are also found in some SLE and SSc patients (3). To compare the disease-specificity of anti-70K and anti-70Kapop autoantibodies, sera from a group of MCTD patients and from a group of patients suffering from a variety of autoimmune disorders were analyzed. As shown in Table 1, we observed that the majority of MCTD patients (54%), displayed antibody reactivities preferentially recognizing the apoptotic form of U1-70K, compared to the intact U1-70K protein. Seven patients (13%) reacted with 70K and 70Kapop with similar efficiencies, and only 6% of the MCTD patients preferentially reacted with the intact 70K protein. Fourteen sera (27%) did not react detectably with either U1-70K polypeptide, although the sera were anti-RNP positive by several techniques. These results indicate that apoptotic 70K is a better antigen for the detection of anti-U1-70K autoantibodies than the intact U1-70K protein. Antibody reactivity with 70Kapop was found in only 2% of sera from control groups, whereas antibody reactivity with 70K was found in 5% of patient sera from control groups.
In some patients with MCTD, antibody titres against the U1 snRNA molecule correlate with disease activity, and could even possess prognostic value (23). In contrast, most studies did not find a correlation between disease activity and antibody responses to 70K, either by serum analysis using recombinant protein as antigen in ELISA (23,24), or by analysis on western blots using native protein from cell extracts (25). Only one study, using ELISA with recombinant U1-70K as technique, has reported decreasing disease activity concomitant with decreasing anti-U1-70K antibody levels (26).

Since apoptotic modifications on autoantigens, such as the cleavage of 70K, are believed to be involved in the primary autoimmune response, we hypothesized that immune complexes containing anti-70K antibodies might also be important for triggering disease flares. Serum samples were longitudinally collected from twelve MCTD patients by a follow-up during variable time intervals (4 – 15 years; average 10 years). All samples were analyzed for the presence of autoantibodies against 70K and 70K>apo on western blots containing non-apoptotic and apoptotic Jurkat cell extracts and the presence of these autoantibodies was compared with the disease activity of each patient. The overall conclusion of this longitudinal study was that no significant correlations between antibody titres against either 70K>apo or 70K and disease exacerbations could be observed.

**Autoantibodies against apoptotic U1-70K are more prevalent early in disease**

As mentioned above, it has been hypothesized that apoptotic modifications trigger the primary immune response towards self-proteins, and that, through secondary immune responses and epitope spreading, autoantibodies directed against unmodified regions on the autoantigen appear at later stages of the disease. To investigate this possibility for the U1-70K autoantigen, the longitudinal serum collection (23) of twelve MCTD patients was re-examined, now for...

<table>
<thead>
<tr>
<th>Patient group (disease)</th>
<th>Nr of patients</th>
<th>70K&gt;apo</th>
<th>70K&gt;apo &gt; 70K</th>
<th>70K&gt;apo = 70K</th>
<th>70K&gt;apo &gt; 70K</th>
</tr>
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<tbody>
<tr>
<td>MCTD</td>
<td>53</td>
<td>15 (28%)</td>
<td>14 (26%)</td>
<td>7 (13%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>SLE</td>
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<td>0</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>non-SLE*</td>
<td>61</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (3%)</td>
</tr>
</tbody>
</table>

* The control group of non-SLE patients consists of patients suffering from PM/DM (n=26), pSS (n=18), SSC (n=10), RA (n=3), RP (n=3) and UCTD (n=1).
antibodies to U1-70K and for antibodies to 70K\(^{\text{apo}}\). Three patients produced antibodies reacting strongly with 70K\(^{\text{apo}}\), whereas no or only weak reactivity against 70K was observed. In one of these three patients autoantibodies against 70K\(^{\text{apo}}\) were more prevalent in early serum samples and the level decreased in time. Eight patients were found to have high titres of antibodies with reactivities to both 70K and 70K\(^{\text{apo}}\). Interestingly, in three of these patients early serum samples demonstrated a higher reactivity with 70K\(^{\text{apo}}\) than with 70K, whereas later samples showed comparable reactivities with both antigens, or higher reactivities with 70K. An example of this type of reactivity profile is shown in Figure 2. One of the twelve patient sera did not detectably contain antibodies directed to 70K or 70K\(^{\text{apo}}\). These results thus support the idea that antibodies to 70K\(^{\text{apo}}\) appear earlier in the disease than antibodies to the complete 70K protein.

**Discussion**

Greidinger et al. recently showed that antibodies to the U1-70K protein in RNP positive patients are often accompanied by antibodies directed to the apoptotic cleavage product of this autoantigen, and that the B cell epitopes recognized on the apoptotic product are antigenically different from those contained in the intact form of the U1-70K protein (18,19). This study is the first to confirm and extend these findings and strongly suggests that the reactivity of a patient serum with anti-U1-70K antibodies depends on the presence of antibodies against epitopes shared by 70K and 70K\(^{\text{apo}}\), and antibodies against epitopes exclusively present on 70K\(^{\text{apo}}\). The major apoptosis-specific epitope on U1-70K has been shown to be located in the N-terminal 205 amino acids, indeed was able to induce an anti-70K\(^{\text{apo}}\) antibody response in mice, with subsequent epitope spreading. Interestingly, some of the immunized mice developed pulmonary lesions comparable to lesions found in lungs of MCTD patients.

In our study, a minority of MCTD sera (4%) contained autoantibodies exclusively reacting with intact 70K. We suggest that these sera derive from patients in a relatively late disease phase and primarily contain antibodies resulting from expanded epitope spreading. The majority of epitopes recognized by these sera might therefore be dependent on the C-terminal part of the protein, which is cleaved off during apoptosis and is not present on 70K\(^{\text{apo}}\). Patients that tested negative in our western blot experiments may either have low levels of anti-70K antibodies, or may not at all produce such antibodies. Instead, other components of the U1 snRNP, such as the U1 RNA molecule, U1A or U1C, may be targeted by these sera and may explain their anti-U1 snRNP reactivity.

We demonstrate here that the majority of U1 snRNP positive patient sera preferentially recognize the apoptotic form of U1-70K, which is most likely explained by the presence of antibodies targeting an apoptotic U1-70K epitope. These results are in line with reports by Greidinger et al., who found that approximately 50% of their RNP positive sera contained apoptotic U1-70K autoantibodies (18,19).

How disease flares are induced is not completely understood. Correlations between serum levels of certain autoantibodies and disease activity have been reported for MCTD and SLE (23,27), but it can be disputed whether these antibodies contribute to the disease flares or are merely epiphenomena. Our data show that antibodies against the apoptotic form of U1-70K do not significantly correlate with disease activity, suggesting that there is no important role for apoptotic U1-70K in the initiation of disease flares. However, it is possible that the variations in antibody levels against the apoptosis-specific epitope are masked by the presence of antibodies against other epitopes on U1-70K and U1-70K\(^{\text{apo}}\). Furthermore, a polyspecific secondary antibody was used to detect bound serum antibodies, and as a consequence variations in isotype-specific antibody levels might have remained undetected.

It has been reported that the first autoantibodies to appear in anti-RNP positive patients are generally antibodies against U1-70K (16,26). Our results suggest that the

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**Figure 2. Longitudinal anti-70K analysis of patient T2.** Eighteen serum samples taken over a period of 7 years with approximately equal time intervals were analyzed on western blots containing non-apoptotic and apoptotic Jurkat cell extracts. The positions of U1-70K, of which two isoforms are visible, and 70K\(^{\text{apo}}\), are indicated on the left. In lane 19, U1-70K was detected with mouse monoclonal 2.73, which reacts much stronger with U1-70K than with 70K\(^{\text{apo}}\).
apoptotic form of U1-70K drives the primary autoimmune response to U1-70K, because in several patients antibodies against an epitope associated with the apoptotic form of U1-70K precede the appearance of reactivity with intact U1-70K. The fact that the first serum samples from relatively few patients exclusively contain anti-70K<sup>apo</sup> antibodies may be due to the stage of disease development at which the patient enters the rheumatologic clinic. It is likely that the first symptoms, later followed by the diagnosis of the disease, had been established years before the start of the longitudinal study. Moreover, it is possible that autoantibodies, especially those that are generated by the primary immune response, were already present before the manifestation of clinical symptoms, and that subsequent epitope spreading might have occurred before the patient entered the rheumatology clinic. For example, anti-cyclic citrullinated peptide (anti-CCP) autoantibody is a very specific symptom (16,28,29). In our opinion this may explain why a relative enrichment of anti-U1-70K antibodies could not be detected in the early sera of all patients.

During apoptosis, the U1 snRNP complex is modified in several ways. In addition to cleavage of U1-70K, U1 snRNA and the Sm-F protein are cleaved, and phosphorylated serine-arginine proteins associate with the complex (30). Apoptotic modifications of the U1A and U1C proteins have not been described so far. U1-70K can be cleaved by caspase-3 and granzyme B, and it can be oxidatively fragmented in the presence of metals, resulting in products of 40, 60 and 55 kDa, respectively. Correlations between recognition of specific U1-70K fragments and disease manifestations are interesting. For example, patients suffering from Raynaud’s phenomenon preferentially recognize the oxidatively modified 55 kDa fragment of U1-70K (31). The findings that early MCTD sera are enriched for antibodies against the 40 kDa apoptotic fragment (70K<sup>apo</sup>) and that most sera show a higher reactivity with this fragment, suggest that caspase-3 cleaved U1-70K plays a role in breaking tolerance in these patients. Although granzyme B is postulated to play a role in breaking tolerance (32) to U1-70K, it is unknown whether specific patient groups preferentially recognize the 60 kDa cleavage product generated by granzyme B, which would be interesting to study in more detail.

Summary

Analysis of a group of MCTD patient sera by western blotting demonstrated that the majority of patient sera recognized the apoptotic form of U1-70K more efficiently than the intact form of the U1-70K protein. The fact that the presence of these antibodies in most patients precedes the occurrence of other anti-U1-70K antibodies suggests that 70K<sup>apo</sup> is particularly important for the early detection of this disease in patients.

Materials and Methods

Patient sera

All patients were seen at the Department of Rheumatology of the University Medical Centre Nijmegen or the St. Maartenskliniek Nijmegen (the Netherlands), and were classified according to standard criteria for each disease. All MCTD patients (n=26) tested positive for anti-U1 snRNP autoantibodies by counterimmunoelectrophoresis, and for antibodies to one or more components of the U1 snRNP complex by immunoblotting. Most of the sera (91%) were also RNP positive as demonstrated by U1 snRNA co-immunoprecipitation. Longitudinal serum collections were obtained from 12 MCTD patients and have been described before (23). From each patient, over a period of 4 to 15 years (average 10 years) 8 to 33 serum samples (average 18 samples) were available and analyzed. During the follow-up study, the patients were regularly monitored for clinical and serological parameters. At each visit, the disease activity was measured according to a validated SLE disease activity index described by ter Borg et al. (27). Medication was given as indicated by the clinical status. Additionally, patient sera were collected from SLE (n=48), PM/DM (n=26), primary Sjögren’s syndrome (pSS) (n=18), SSc (n=45), RA (n=3), Raynaud’s phenomenon (RP) (n=3) and undefined connective tissue disease (UCTD) (n=4). Informed consent was obtained from all participants according to the medical ethical regulations of the local ethics committee. Sera were stored at −70°C until use.

Cell lines, induction of cell death and preparation of cell extracts

Jurkat (human T cell leukemia) suspension cells were grown in RPMI 1640 medium (Gibco-BRL), supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin and 10% heat-inactivated foetal calf serum (Gibco-BRL), in a humidified 37°C incubator containing 5% CO<sub>2</sub>. Cells were maintained at a concentration of 10<sup>5</sup> cells/ml and were induced to undergo apoptosis by addition of 10 µg/ml anisomycin. Eight hours after induction, apoptotic cells were harvested by centrifugation at 800g for 10 minutes and washed with PBS. Apoptotic and non-apoptotic Jurkat cells were resuspended in Nonidet-P40 (NP40) containing lysis buffer (25 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 0.25 mM diethytherit (DTE), 1% NP40, Complete<sup>™</sup> protease inhibitor cocktail (Roche)) at a concentration of 1x10<sup>6</sup> cells/ml. Cells were lysed on ice for 30 minutes and subsequently centrifuged for 30 min at 12,000g and 4°C. Supernatants were used immediately or stored at −70°C.

SDS-polyacrylamide gel electrophoresis and western blotting

Cell extracts of 1x10<sup>5</sup> non-apoptotic Jurkat cells and 1x10<sup>5</sup> apoptotic Jurkat cells, either separately or mixed, were separated by SDS-PAGE. Directly after gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher&amp;Schuell) by semi-dry electroblotting. Ponceau S (Sigma) staining of the membrane was used to verify protein transfer.

Probing western blots with patient sera

All incubation steps were carried out at room temperature on a shaking table. Western blots containing non-apoptotic and apoptotic Jurkat cell extracts were pre-blocked with 5% non-fat dried milk in PBS containing 0.1% Nonidet-P40 (MPBS/ NP40) for two hours. Subsequently, membranes were incubated with patient serum, 1,000- to
Autoantibodies to apoptotic U1-70K

5,000-fold diluted in MPBS/NaPO4, for one hour. After extensive washing with PBS containing 0.1% NaPO4 (PBS/NaPO4) membranes were incubated with horseradish peroxidase (HRP)-labelled rabbit anti-human IgA/IgG/IgM antibody (DAKO), 1,000-fold diluted in MPBS/NaPO4, for one hour. After several washes with PBS/NaPO4 and PBS, bound antibodies were detected by enhanced chemiluminescence (ECL). Antibody reactivities against 70K and 70K" were scored ranging from 0 to 5 by three researchers independently. In each experiment several control antibodies were used.

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Chapter 7

**Apoptotic modifications of the nuclear proteins hnRNP C, p54\textsuperscript{nrb} and PSF**

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Recently, we isolated recombinant antibodies directed to the nuclear proteins hnRNP C, p54nrb and PSF from autoimmune libraries by subtractive selection on apoptotic cell extracts. In the study presented here, the apoptotic modifications on these proteins were studied by two-dimensional IEF/SDS-PAGE immunoblotting and indirect immunofluorescence. It was demonstrated that p54nrb was not only cleaved during apoptosis, as described before, but that the isoelectric point changed from pI 6-7 in normal cells to pI 8-9 in apoptotic cells. Also the hnRNP C1 protein demonstrated modifications in addition to the previously reported apoptotic cleavage. Five isoforms of hnRNP C1 were present in the normal cell extract, whereas only three isoforms were detected in the apoptotic cell extract. PSF and hnRNP C were demonstrated to translocate to apoptotic bodies on the cell surface, as has been shown previously for several other autoantigens. In addition, we analyzed the presence of autoantibodies directed to recombinant p54nrb in sera from patients suffering from systemic lupus erythematosus (n=65), systemic sclerosis (n=79), polymyositis/dermatomyositis (n=24), polymyositis/scleroderma overlap syndrome (n=24), and mixed connective tissue disease (n=12). Autoantibody reactivity to p54nrb was detected in 8% (2/24) of the PM/Scl patients, and none of the other sera. Our results corroborate the idea that apoptotic modifications create neo-epitopes on autoantigens as PSF, p54nrb and hnRNP C1, which consequently can break tolerance to these self-proteins.

Introduction

Heterogeneous nuclear ribonucleoprotein C (hnRNP C) is a protein abundantly expressed in all eukaryotic cells. Two variants of hnRNP C (C1 and C2) polypeptides exist, resulting from alternative splicing leading to a 13 amino acid insertion after position 107 in hnRNP C2 (1). The protein is located in the nucleus, excluding the nucleoli (2), and is one of the most abundant nuclear proteins (3,4). In vivo and in vitro, hnRNP C forms heterotetramers of three C1 molecules and one C2 molecule (5). Recently, the oligomerization has been visualized using nuclear magnetic resonance (6). Three heterotetramers form a 198 triangular complex that binds approximately 700 nucleotides of RNA (7), and these 198 complexes are part of a larger 408 complex containing other hnRNPs (8). As schematically depicted in Figure 1, four distinct domains can be identified in hnRNP C1 and C2, namely an RNA recognition motif (RRM), a basic domain involved in binding pre-mRNA, a leucine zipper mediating monomer-monomer interactions, and an acidic carboxyterminal

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domain. HnRNP C is involved in pre-mRNA packaging, spliceosome assembly and nuclear retention of unspliced hnRNA (9). It functions in actively packaging and chaperoning nascent pre-mRNA transcripts in 40S hnRNP particles. In addition to the known involvement of hnRNP C in mRNA binding, new functions of the protein recently became apparent: hnRNP C has been described to have a role in viral RNA replication (10-13), repair of DNA damage (14), cell-cycle control (15,16) and telomere function (17).

The hnRNP A1, A2, B1, B2, C1 and C2 proteins are the so-called core proteins of hnRNP complexes, and these six proteins are highly related, with molecular weights ranging from 34 to 43 kDa (see review by (18)). Autoantibodies directed to hnRNP A1 and hnRNP A2/B (also named anti-Ra33 autoantibodies) are found in a large number of patients suffering from systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD) (see review by (18)). Autoantibody reactivity with hnRNP C, however, has been described in only a few patients suffering from various autoimmune diseases. In 1997, Stanek et al. were the first to describe the presence of autoantibodies directed to hnRNP C, and they found this reactivity in one patient with the atypical coexistence of systemic sclerosis (SSc, also referred to as scleroderma) and psoriatic arthritis (19). In 2000, Heegaard et al. described three patients, which they selected based upon an unusual anti-nuclear antibodies staining pattern, with anti-hnRNP C autoantibody reactivity. One patient suffered from arthralgias and oligoarthritis, one patient had complaints of muscle tenderness, and one patient suffered from polymyositis in addition to psoriasis (20). Recently, we isolated a recombinant antibody (single chain variable fragment, scFv) from autoimmune libraries derived from SLE, SSc, and RA patients by subtractive selection from apoptotic cell extract (21). In the same subtractive selection, an additional scFv was obtained directed to both nuclear RNA-binding protein of 54 kDa (p54nrb) and polypyrimidine tract-binding protein (PTB) (22). Both proteins bind the polypyrimidine tract, which is a region within introns that is important for the definition of the 3'-splice site. Later it became clear that the majority of PSF molecules in the cell are bound to the nuclear matrix and that only a minority is bound to PTB (23). PSF is distributed in the nucleoplasm, both diffuse and in punctuate patterns. As schematically illustrated in Figure 2, the p54nrb protein is highly homologous to the C-terminus of PSF (24). PSF and p54nrb were identified as a p100/p52 DNA-binding heteromer (25), and indeed both proteins bind dsDNA, ssDNA, and RNA, and are involved in a wide variety of cellular processes such as nuclear RNA splicing, regulation of transcription, DNA unwinding and DNA pairing, and nuclear retention of defective RNAs (see review by (26)). So far, autoantibodies directed to neither p54nrb nor PSF have been described to occur in patient sera.

It is still not understood why autoimmune patients produce antibodies to self-antigens (autoantigens), but an increasingly recognized hypothesis focuses on the role of autoantigen modifications, especially those occurring during cell death, in breaking immunological tolerance to self-antigens (see reviews by (27-30)). It is believed that these modifications, as for example cleavage, phosphorylation and citrullination, create neo-epitopes on autoantigens that are not recognized by the immune system as ‘self’ and therefore initiate a primary immune response. Secondary immune responses subsequently lead to autoantibodies that recognize other (non-modified) epitopes on the same molecule or complex. This process is called epitope spreading (31,32). HnRNP C, p54nrb and PSF have been described to be modified during apoptosis. HnRNP C1 and C2 are cleaved by caspase-3, leading to the removal of a peptide of approximately 2 kDa (33). The p54nrb protein is also cleaved, resulting in a fragment of approximately 50 kDa (34). PSF is known to be hyper-phosphorylated during apoptosis (35), and this modification is thought to occur on serine, and to a lesser extent threonine, residues residing in its N-terminal part. In this study the apoptotic modifications of hnRNP C, p54nrb and PSF were further studied by immunoblotting of...
two-dimensional isoelectric focusing/SDS-PAGE (2D) gels using specific scFvs, and their localization in apoptotic cells was examined by indirect immunofluorescence. We also addressed the question whether p54nrb and PSF are autoantibody targets by analyzing patient sera from different autoimmune diseases.

Results

Apoptotic modifications of hnRNP C, p54nrb and PSF

To induce apoptosis, Jurkat cells were incubated with anisomycin for eight hours, as described previously (36), and cell extracts were prepared by sonication followed by centrifugation. Cleavage of the U1-70K protein, a component of the U1 small nuclear ribonucleoprotein (U1 snRNP) complex, is regarded as a biochemical marker for apoptosis (37). In the apoptotic Jurkat cell extract the U1-70K protein (70 kDa) was completely cleaved to the characteristic 40 kDa apoptosis-specific fragment, (data not shown), confirming the efficient induction of apoptosis by this procedure. The proteins in the apoptotic and non-apoptotic cell extracts were separated by isoelectric focusing (IEF) in the first dimension and by SDS-PAGE in the second dimension. After western blotting, p54nrb and PSF were detected with scFv B1H11, and hnRNP C1 and C2 were detected using scFv G1G10 (21). As shown in Figure 3a, many isoelectric isomers of the 100 kDa protein PSF were observed with isoelectric points ranging from approximately pH 4 to pH 10. The most intense signals were observed in the basic region of this pH range.

No significant differences were observed when the patterns of PSF isoforms in the normal and apoptotic cell extracts were compared.

On the western blot with normal cell extract, p54nrb was detected in six spots at 54 kDa in the pH range 6 to 7. The four additional spots that were detected at approximately 52 kDa in the pH range 5 to 6 probably represent splicing variants of p54nrb, although the possibility that these isoforms result from posttranslational modifications cannot be excluded. On the western blot containing apoptotic cell extract only four spots were detected at the 54kDa/pH 6-7 location. Because the relative intensity of these spots was significantly reduced compared to the non-apoptotic material, the amount of these isoforms seems to be reduced during apoptosis, which may be due to proteolytic cleavage. Simultaneously, at least five new p54nrb spots were detected at approximately 50 kDa and at a pH range 8 to 9, as well as a number of faster migrating isoforms in the same pH range. These spots are most likely generated via apoptotic cleavage of p54nrb, as has been described before (34). These data indicate that besides cleavage also the isoelectric nature of p54nrb shows marked changes in apoptotic cells.

By running an 8% polyacrylamide gel in the second dimension, it was possible to separate hnRNP C1 from hnRNP C2 (Figure 3b). The stronger signal of the smaller isoform C1 is consistent with the composition of hnRNP C heterotetramers, containing three hnRNP C1 molecules and one hnRNP C2 molecule. The apoptotic forms of hnRNP C1 and C2 run slightly faster than those in normal cell extracts, which is caused by the removal of a fragment of approximately 2 kDa in apoptotic cells (33). On the 2D western blot with normal cell extract five hnRNP C1 and three hnRNP C2 spots were detected. The corresponding blot containing apoptotic cell extract showed three spots for both hnRNP C1 and hnRNP C2, indicating that during apoptosis hnRNP C1 undergoes additional modifications besides cleavage. Moreover, the intensity of the three spots in the apoptotic material is very similar, whereas in the normal cell extract two spots are far more intense than the other spots (both for hnRNP C1 and hnRNP C2).
HnRNP C and PSF translocate to apoptotic bodies on the cell surface

During apoptosis, several autoantigens translocate from their normal cellular location to apoptotic blebs and apoptotic bodies on the cell surface (38,39). Due to this translocation (modified) autoantigens may be more easily exposed to the immune system. To study the localization of hnRNP C and PSF during apoptosis, monolayer HEp-2 cells were exposed to anisomycin for six hours. Subsequently, the localization of hnRNP C and PSF during apoptosis was detected to anisomycin for six hours. Subsequently, the localization of hnRNP C and PSF during apoptosis was detected (modified) autoantigens may be more easily visualized (40). Therefore, other antibodies to these proteins were used in this experiment: hnRNP C was detected with patient serum BH, which contains antibodies with high specificity and affinity for hnRNP C (19); PSF was detected using monoclonal antibody B92; and for p54<sup>np</sup> no alternative antibody was available. As a control, cells were incubated only with the secondary antibody to [polyclonal antibody](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5540512/). As mentioned above, autoantibody reactivity directed to hnRNP C has been described in a few patients (19,20), and scFvs directed to hnRNP C were found to be translocated to the apoptotic bodies (Figure 4b and 4c, respectively).

**The p54<sup>np</sup> protein is recognized by polymyositis/scleroderma overlap patients**

As mentioned above, autoantibody reactivity directed to hnRNP C has been described in a few patients (19,20), and scFvs directed to hnRNP C could be isolated from antibody phage display libraries derived from autoimmune patients (21). Serum autoantibody reactivity directed to PSF or p54<sup>np</sup>, on the other hand, has not been described so far. However, the apoptotic modifications of p54<sup>np</sup> and PSF described here, the translocation of PSF to apoptotic bodies, and the isolation of the anti-p54<sup>np</sup>/PSF recombinant antibody from autoimmune antibody libraries, suggest that p54<sup>np</sup> and/or PSF are also genuine autoantigens. To detect this autoantibody reactivity in patient sera, we analyzed groups of patients with different autoimmune diseases for autoantibody reactivity with p54<sup>np</sup> by immunoblotting.

Because the anti-PSF/p54<sup>np</sup> scFv B1H11 was isolated from a mixture of autoimmune libraries, including libraries from SLE (n=5) and SSc (n=5) patients, autoantibody reactivity with these proteins was expected to be present in sera from these diseases, or in sera from the related autoimmune diseases polymyositis/scleroderma overlap disease (PM/Scl) and MCTD. PM/Scl patients demonstrate symptoms from polymyositis/sclerodermopathy (PM/DM) and scleroderma, and MCTD patients show a combination of symptoms that are typically associated with SLE, SSc, RA, and PM/DM (40). Because of the PM/DM symptoms in these two patient groups, a group of PM/DM patients was analyzed as well.

To develop a specific assay for anti-PSF/p54<sup>np</sup> detection, cDNAs encoding both proteins were expressed in E.coli. Since only p54<sup>np</sup> was expressed to high levels in this system, PSF was not included in the screening for autoantibody reactivities. The recombinant p54<sup>np</sup> was purified by ammonium sulphate precipitation, followed by anion exchange chromatography and cation exchange chromatography. The purity of recombinant p54<sup>np</sup> was at least 90%, as demonstrated by silver-staining of SDS-PAGE gels (data not shown). Sera from patients suffering from SLE (n=65), SSc (n=79), PM/DM (n=24), PM/Scl (n=24), and MCTD (n=12), were analyzed for reactivity with recombinant p54<sup>np</sup>. As demonstrated in Figure 5, two PM/Scl sera (Day5 and Myo36), but none of the other patient sera reacted with p54<sup>np</sup>. This finding shows that p54<sup>np</sup> indeed is an autoantigen in PM/Scl patients, although the frequency by which these autoantibodies occur in such patients seems to be low (about 8%).

**Figure 4. Immunofluorescence detection of antigens in apoptotic HEp-2 cells.** HEp-2 cells were incubated with 10 μg/ml anisomycin for 6 hours, and cells were fixed with methanol (a and b) or p-formaldehyde (c). Fluorescence images (at the left) and phase-contrast (a and b) or bright-field (c) images (at the right) are shown for each experiment. (a) Detection of the U1 snRNP complex, hnRNP C, and PSF in apoptotic cells. The U1 snRNP complex was detected with patient serum D18, which is reactive with the U1-70K and U1A proteins. The U1-70K protein has been reported to localize in a rim around the nucleus relatively early during apoptosis, and later in apoptotic bodies on the cell surface (38). Both the rim and the bodies at the cell surface were indeed observed with patient serum D18 (Figure 4a). The recombinant scFv antibodies to hnRNP C, and to p54<sup>np</sup> and PSF did not give a clear signal in the immunofluorescence assay, which may be either due to sensitivity problems or to inaccessibility of the epitopes recognised by these monoclonal antibodies. Therefore, other antibodies to these proteins were used in this experiment: hnRNP C was detected with patient serum BH, which contains antibodies with high specificity and affinity for hnRNP C (19); PSF was detected using monoclonal antibody B92; and for p54<sup>np</sup> no alternative antibody was available. As a control, cells were incubated only with the secondary antibody to [polyclonal antibody](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5540512).

**Figure 5. Antibody reactivity of patient sera Day5 and Myo36 with recombinant p54<sup>np</sup> on immunoblot.** The position of recombinant p54<sup>np</sup> on the western blots (indicated with an arrow at the right) was visualized by Ponceau S staining. The blots were incubated with patient sera and bound antibodies were detected with HRP-labelled goat antimouse IgG/C/M antibodies and ECL. In total 24 PM/Scl sera were analysed, but only the data for 11 sera are shown. Patients Myo36 (lane 1) and Day5 (lane 9) reacted with a protein at the position of p54<sup>np</sup>. The positions of molecular mass markers are indicated on the left.
Discussion

The nuclear protein hnRNP C has been described before as an autoantigen in several patients with different autoimmune diseases, and we demonstrate here that p54\textsuperscript{47}, another protein that was identified as a target of autoimmune patient derived scFv antibodies, indeed is also recognized by autoantibodies in patient sera. Furthermore, the additional experimental data on the modifications and translocations of p54\textsuperscript{47}, PSF and hnRNP C during apoptosis support the hypothesis that apoptotic modifications are involved in breaking of immunological tolerance to these autoantigens in susceptible individuals.

In 2001, Shav-Tal \textit{et al.} reported that PSF is hyperphosphorylated during apoptosis (35). However, the 2D immunoblots that we presented here did not demonstrate significant changes in the isoelectric point of the 100 kDa PSF isoforms, which would be expected as a result of hyperphosphorylation. These different observations might be due to differences in cell lines: Shav-Tal \textit{et al.} used HeLa cells, whereas Jurkat T cells were used in the study presented here. Similarly, a dependence of autoantigen modification on the type of tissue or cell has been described previously for the scleroderma autoantigen B23 (41). In contrast, marked differences were observed between the pI values of the apoptotic fragments of p54\textsuperscript{47} (several spots at pI 8 to 9) and those of the intact forms (several spots at pI 6 to 7). These differences might result from losing the part of the protein that is cleaved off, although this is unlikely since neither the N-terminal nor the C-terminal ends of p54\textsuperscript{47} contain clusters of negatively charged amino acids. The change in pI could also be caused by other apoptotic modifications, but more experiments are needed to investigate this possibility. An equivalent change in pI was not observed in a proteomics study by Thiede \textit{et al.}, where the apoptotic cleavage of p54\textsuperscript{47} was first described (34). Since this study was not aimed at the detection of apoptotic variants of p54\textsuperscript{47}, the 2D spots representing these apoptotic fragments of p54\textsuperscript{47} may have escaped the attention of these investigators.

HnRNP C\textsubscript{1} and \textsubscript{2} are known to be cleaved by interleukin 1b-converting enzyme-like proteases during apoptosis (33), but the finding presented here that apoptosis also leads to changes in the isoelectric isoforms indicates that additional apoptotic modifications occur on hnRNP C. The five spots obtained with material from normal cells probably represent the four phosphorylation states that are described for hnRNP C and its non-phosphorylated state (42,43). On the 2D blot containing apoptotic cell extract, hnRNP C\textsubscript{1} was separated in three different spots. As depicted in Figure 1, phosphorylation of hnRNP C\textsubscript{1} takes place on Ser\textsuperscript{247}/Ser\textsuperscript{255}, Ser\textsuperscript{241}, Ser\textsuperscript{242}, and Ser\textsuperscript{240} (42,43). Putative caspase cleavage sites have been appointed to positions Asp\textsuperscript{256}, Asp\textsuperscript{258}, Asp\textsuperscript{264}, Asp\textsuperscript{269}, and Asp\textsuperscript{263} (35). Cleavage at one of these positions would lead to the removal of one phosphorylated serine residue (Ser\textsuperscript{256}). It could be hypothesized that one of the remaining three phosphorylated serine residues of hnRNP C\textsubscript{1} is in addition dephosphorylated during apoptosis. Dephosphorylation in apoptotic cells indeed can occur, as has been described for the La protein (44).

A detailed characterization of the hnRNP C isoforms is required to investigate whether the different spots are due to a different number of phosphate groups on the protein, or whether other modifications are involved.

PSF has been previously reported to localize to apoptotic bodies using a GFP-PSF fusion construct (35). The immunofluorescence data presented in this study, using a monoclonal antibody to monitor the subcellular localization of PSF, are clearly in line with this report. The redistribution of hnRNP C to apoptotic bodies has not been previously described, and suggests that also this nuclear protein can be more easily exposed to the immune system as a result of apoptosis.

We report here for the first time the detection of autoantibodies directed to p54\textsuperscript{47} in patient sera. Upon screening different groups of autoimmune patients, it was found that 8% (2/24) of patients with PM/Scl contain this autoantibody. To demonstrate that autoantibody reactivity with p54\textsuperscript{47} is specific for PM/Scl, and can thus be regarded as a (minor) disease marker, it would be valuable to analyze a larger cohort of PM/Scl patients, in addition to more control groups of autoimmune diseases, inflammatory diseases and healthy individuals. Importantly, the scFv reactive with this protein was isolated from a mixture of libraries not including a PM/Scl patient, substantiating the need for analyses with larger cohorts of sera. Serum reactivity with PSF could not be determined, due to limitations in the expression of this relatively large protein in \textit{E. coli}. Patient sera with autoantibodies to p54\textsuperscript{47} might be expected to react with PSF as well, since p54\textsuperscript{47} is highly homologous to the C-terminus of PSF (24). The fact that the scFv antibody B1H11 reacts with an epitope that is present on both PSF and p54\textsuperscript{47} suggests that such autoantibodies indeed exist in autoimmune patients.

We hypothesize that the apoptotic modifications on hnRNP C, p54\textsuperscript{47} and PSF lead to the generation of neo-epitopes. Upon their translocation to the surface of apoptotic cells, these neo-epitopes are more easily exposed to the immune system, and are thus more likely to trigger a primary autoimmune reaction. Experimental data that would strongly support a role for neo-epitopes in the autoantibody formation would be the detection of autoantibodies that only react with the apoptotic variants of p54\textsuperscript{47}, PSF and hnRNP C.

Such apoptosis-specific autoantibodies are for example described for the MCTD autoantigen U1-70K (39,45,46), and the La protein, which is an autoantigen in Sjögren’s syndrome (47). Autoantibodies to the apoptotic form U1-70K (the 40 kDa fragment that results from cleavage by caspase-3) have been demonstrated to be present especially during the early phases of the disease (36). Evidence for the early presence of apoptosis-specific autoantibodies may be difficult to obtain in view of the small number of sera reactive with hnRNP C and p54\textsuperscript{47} and the fact that patient sera are generally collected in later stages of the disease.
Materials and Methods

Cell lines

Jurkat (human T cell leukemia) suspension cells were grown in RPMI 1640 medium (Gibco-BRL), supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin and 10% heat-inactivated fetal calf serum (Gibco-BRL). HEp-2 cells were cultured in DMEM medium (Gibco-BRL), supplemented with 10% heat-inactivated FCS, 1 mM penicillin, and 1 mM streptomycin. Cells were grown in a humidified 37°C incubator containing 5% CO₂.

Induction of cell death and preparation of cell extracts

Jurkat cells were maintained at a concentration of 10⁶ cells/ml. For induction of apoptosis, cells were incubated in the presence of 10 µg/ml anisomycin for 8 hours at 37°C. Cells were harvested by centrifugation at 800 x g for 5 minutes, and washed twice with PBS. Cells were then resuspended in ice-cold sonication buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.05% Nonidet-P40, 1 mM EDTA, 1 mM DTT, and Complete™ protease inhibitor cocktail (Roche)) and lysed by sonication on ice. After centrifugation at 12,000 x g and 4°C for 20 minutes, clear lysates were used immediately or stored at −70°C.

Patient sera and antibodies

Patient sera were present in the serum collection of the Department of Biochemistry, Radboud University Nijmegen (The Netherlands). These patients had been seen at the St. Maartenskliniek Nijmegen or at the Department of Rheumatology of the University Medical Centre Nijmegen (The Netherlands). Patients had been classified in accordance with standard criteria for each disease, and informed consent was obtained from all participants. Patient sera were present from systemic lupus erythematosus (n=65), systemic sclerosis (n=79), polymyositis/dermatomyositis (n=24), polymyositis/scleroderma overlap syndrome (n=24), and mixed connective tissue disease (n=12). Control sera from healthy individuals (n=30) were present as well. Sera were stored at −70°C until use. Patient serum BH with known reactivity with hnRNP C was a kind gift from J. Vencovsky (Charles University, Prague, Czech Republic). Recombinant antibodies directed to p54⁴⁴α/PSF and hnRNP C had been previously selected from autoimmune libraries in a subtractive antibody phage display selection procedure on apoptotic cell extract (21). Recombinant antibodies were C-terminally tagged with a VSV-G- and a 6XHis-tag, expressed in E.coli and isolated from the periplasm as described previously (48).

Protein expression and purification

Expression of p54⁴⁴α from pET-9c in E.coli resulted in the efficient production of mainly insoluble protein. In order to increase the solubility of recombinant p54⁴⁴α, the expression was performed in the presence of 3% ethanol as described (49). In short, LB medium containing 70 µg/ml kanamycin was inoculated with the bacterial stock and grown overnight at 37°C and 200 rpm. The overnight culture was diluted 100-fold in LB medium containing 70 µg/ml kanamycin and 3% ethanol, and grown for 6 hours at 30°C and 200 rpm. Protein expression was induced by the addition of 1 mM IPTG, and subsequently the bacterial culture was grown overnight at 30°C and 200 rpm. The bacterial culture was centrifuged at 4,000 x g and 4°C for 15 minutes, resuspended in one-tenth of the starting volume lysis buffer (25 mM Tris-HCl pH7.6, 25 mM NaCl, 1 mM EDTA, 1 mM DTT), and sonicated on ice. After centrifugation at 4,000 x g and 4°C for 30 minutes, the supernatant contained the soluble p54⁴⁴α protein, which was then purified by a combination of chromatographic and precipitation steps. The protein was first separated from other proteins by ammonium sulphate precipitation. Subsequently, p54⁴⁴α, which precipitated at 20% ammonium sulphate, was dissolved, dialyzed and separated from other contaminating proteins by anion exchange chromatography on a DEAE column (GE Healthcare). SDS-PAGE analysis revealed that the fractions containing p54⁴⁴α still contained relatively high levels of other proteins. These fractions were pooled, and finally separated by cation exchange chromatography on a Source 15S column (GE Healthcare). The purity of p54⁴⁴α in the fractions of this column was approximately 90%, as estimated from a silver-stained SDS-PAGE gel.

Gel electrophoresis and immunoblotting

For two-dimensional gel electrophoresis, proteins from apoptotic and non-apoptotic Jurkat cell extracts were separated by isoelectric focusing (IEF), followed by separation by SDS-PAGE. First, salts and other substances interfering with IEF were removed using a clean-up procedure (2D Clean-up Kit, GE Healthcare). Proteins were separated on 13 cm Immobiline™ DryStrip gel (GE Healthcare) with a pH range 3-10, or pH range 4-7, using the IPGphor II Isoelectric Focusing System (GE Healthcare). After IEF, the gel strips were incubated in equilibration buffer (50 mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, 10 mg/ml DTT, and bromophenol blue). For separation on molecular weight in the second dimension, IEF strips were placed on SDS-PAGE gels. For one-dimensional SDS-PAGE separation, SDS-PAGE loading buffer was added to protein samples, and samples were incubated at 95°C for 5 minutes. After one- or two-dimensional gel electrophoresis, proteins were transferred to nitrocellulose membranes by semi-dry Western blotting. Protein transfer was checked with a Ponceau S staining. All immunoblotting steps were carried out on a shaking table at room temperature. Membranes were blocked with 5% non-fat dried milk powder in PBS containing 0.05% Tween-20 (MPBST). Recombinant antibodies were incubated with the membrane at a 10- to 20-fold dilution in MPBST, and bound antibodies were detected with anti-VSV-G monoclonal mouse antibodies, 5,000-fold diluted in MPBST, and horseradish peroxidase (HRP)-labelled rabbit anti-mouse antibodies (DAKO), 2,500-fold diluted in MPBST, followed by enhanced chemiluminescence (ECL). Patient sera were incubated with the membrane at a 1,000-fold dilution, and bound antibodies were detected using HRP-labelled goat anti-human IgA/G/M antibodies (DAKO), 1,000-fold diluted, and ECL.

Immunofluorescence analysis on apoptotic HEp-2 cells

For induction of apoptosis, HEp-2 cells were incubated with 10 µg/ml anisomycin for 6 hours. Cells were fixed on glass slides by methanol fixation, or by p-formaldehyde fixation. For methanol fixation, cells were washed with PBS, and incubated with methanol (-20°C) for 10 minutes. After a short wash with acetone, glass slides were air-dried. For p-formaldehyde fixation, cells were washed with PBS, and fixed on glass slides by incubation with 4% p-formaldehyde in 250 mM Hepes, pH 7.4 for 30 minutes on ice. After washing, the cells were incubated with 300 mM glycine in PBS for 5 minutes at room temperature. The slides were washed again with PBS, and subsequently incubated with
Chapter 7

References


Chapter 8

Modification of scleroderma autoantigens by oxidative damage in vitro

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Introduction

Autoimmune diseases are characterized by the presence of high levels of antibodies to self-antigens. Systemic sclerosis (scleroderma) is a relatively rare autoimmune disease, affecting approximately 0.03% of the population of the industrialized world. The pathogenesis of scleroderma is thought to involve oxidative stress and the formation of free radicals, leading to tissue injury and autoantigen formation. It is hypothesized that posttranslational modifications, especially modifications that occur during cell death, create neo-epitopes on autoantigens, which are consequently involved in breaking tolerance to self-antigens. Radicals, produced during oxidative stress, have been put forward as a primary cause of modifications on scleroderma autoantigens. In this study, sera from patients with systemic sclerosis (n=72), Raynaud’s phenomenon (n=129), polymyositis/scleroderma overlap syndrome (n=24), systemic lupus erythematosus (n=5), rheumatoid arthritis (n=4), undifferentiated connective tissue disease (n=5), and mixed connective tissue disease (n=1) were analyzed by immunoblotting for differential autoantibody reactivity with antigens in oxidatively modified and control cell extracts. Five systemic sclerosis patients, five Raynaud’s phenomenon patients, and two patients with polymyositis/scleroderma overlap syndrome were identified that displayed differential autoantibody reactivities. Two of these sera were further analysed using oxidatively modified, apoptotic and necrotic cell extracts, and it was found that the polypeptides recognized by these sera in the oxidatively modified cell extracts were not created during apoptosis and necrosis. These findings further substantiate the idea that oxidative modification of autoantigens is involved in the development of autoimmunity in scleroderma and related disorders.

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phosphorylation, hyperphosphorylation, trans-glutamination, and citrullination (5,6). Furthermore, during apoptosis, the (modified) proteins translocate to apoptotic blebs at the cell surface (7,8), and it is believed that in such a way they are more easily exposed to the immune system. During necrosis, proteins are modified as well (6,9,10), and modified autoantigens may leak out of the cell due to the loss of membrane integrity of the necrotic cell. In a situation of massive cell death and/or a deficiency in the clearance system, the dying cells are not efficiently removed and elevated levels of modified self-molecules, displaying neo-epitopes, are presented to the immune system (11).

Some autoantibodies specifically occur in a particular autoimmune disease, and therefore have clinical relevance (12). For example, autoantibodies directed to the U1 small nuclear ribonucleoprotein complex (U1 snRNP) are associated with systemic lupus erythematosus (SLE) overlap disease (also referred to as mixed connective tissue disease, MCTD) (13). Recently, it was demonstrated that during apoptosis a neo-epitope is formed on the U1 snRNP, more specifically on its U1-70K protein component, and that patient sera contain autoantibodies that specifically recognize granzyme B-cleaved La protein (19).

Much less is known about an association between the other forms of cell death (necrosis and oxidative stress) and autoimmune diseases. Systemic sclerosis (SSc, also referred to as scleroderma) is an autoimmune disease that affects approximately 0.03% of the population of the industrialized world (20). SSc is characterized by excessive production of collagen, which not only occurs in the skin, but also in tissues surrounding organs like lungs, heart, kidneys and gut. The tissue damage in SSc is thought to be caused by free radicals (oxidative stress) (21), which are produced upon ischemia-reperfusion. A short period of ischemia (O₂-depletion), reperfusion of the tissue takes place, and it is thought that in some cases re-oxygenation of the tissue is too rapid, leading to the production of free radicals that cannot be efficiently removed by the tissue. Free radicals stimulate fibroblast proliferation and fibrosis, and cause endothelial injury of micro-vessels leading to vasoconstriction, which leads to further ischemia-reperfusion (22). SSc can be divided into diffuse and limited forms. Diffuse SSc is associated with autoantibodies directed to DNA topoisomerase I (Topo), whereas limited SSc often is associated with autoantibodies to centromere proteins (12). Anti-centromere autoantibodies are associated with higher risk of ischemic digital loss and pulmonary hypertension (12,23). A form of limited SSc is CREST (Calcinosis, Raynaud’s phenomenon, Esophageus dismotility, Sclerodactylly and Telangiectasias). Raynaud’s phenomenon (RP) is characterized by numbness, tingling and/or colour changes in the fingers and toes, which are caused by recurrent episodes of reversible vasospastic attacks of the digital arteries and are triggered by exposure to cold or by emotional stress. Secondary RP is present in ~90% of patients with limited SSc, but RP can also exist without other autoimmune symptoms. A small percentage of such primary RP patients (~9%) develops SSc (24), and these can be regarded as early SSc patients.

A potential mechanism for specific fragmentation of scleroderma antigens has been proposed: metal-catalyzed oxidation by free radicals (25). In vitro, oxidative fragmen-tation is dependent on metal ions capable of supporting Fenton chemistry like copper (Cu²⁺) and iron (Fe²⁺) (Fenton reaction = Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + OH•) (25). A Fenton reaction produces free radicals that can cause fragmentation of the antigen near metal-binding sites (26). Greidinger et al. demonstrated that recognition of cell death-specific fragments by patient sera can correlate with clinical symptoms (27). They demonstrated that SLE patients with RP preferentially recognized oxidatively modified fragments of the U1-70K protein, whereas serum from patients with lupus skin disease preferentially reacted with caspase-cleaved U1-70K. These findings suggested that neo-epitopes generated upon damage by radicals are linked to RP, which in some cases can be considered as an early phase of SSc.

In this study, we analyzed sera from SSc patients for differential autoantibody reactivity to antigens in normal and oxidatively modified Jurkat cell extracts by immunoblotting. Next to patients with SSc, patients suffering from the SSc-related conditions RP and polymyositis/scleroderma overlap syndrome (PM/Sc), and patients suffering from other autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), undifferentiated connective tissue disease (UCTD), and mixed connective tissue disease (MCTD) were analyzed as well.

Results

Oxidative modification of Jurkat cell extracts

Apoptotic pathways have been well characterized in Jurkat T cells, and this cell line has been extensively used for the characterization of apoptotic and necrotic modifications of autoantigens (18,28). To compare autoantibody reactivity directed to polypeptides in oxidatively modified cell extracts with reactivities to autoantigens present in extracts from apoptotic and necrotic cells, the same Jurkat T cell line
was chosen. Oxidative modification can be introduced in vitro by incubation with Fe(II) or Cu(II), either alone or in combination with H₂O₂ or ascorbic acid (25). Cell extracts were prepared by sonication and removal of debris by centrifugation, and were used immediately for oxidative treatment. Since EDTA binds divalent ions, EDTA was omitted from the lysis buffer, and an EDTA-free cocktail of protease inhibitors was used to reduce proteolytic degradation. Various conditions (0.1 mM Fe(II)SO₄, either alone or in the presence of 1.7 mM ascorbic acid or 1 mM H₂O₂; and 0.1 mM Cu(II)SO₄ in combination with 1 mM H₂O₂) (25) were used to introduce oxidative modifications. Modification was monitored by detection of the oxidation-specific 33-38 kDa smear derived from the U1-70K protein (70 kDa), leading to the formation of a smear with a molecular weight of 53-59 kDa (25). Consistent with previous reports the most efficient production of the U1-70K-derived fragments (Figure 1) was observed with 0.1 mM Fe²⁺ and 1.7 mM ascorbic acid at room temperature for 30 minutes (25,27). As a negative control, cell extract was incubated in parallel, but in the absence of Fe²⁺ and ascorbic acid. Polypeptides were visualized by patient serum B6, which was known to react with several components of the U1 snRNP, including U1-70K, U1A, and Sm-B/B’ (18).

**Screening patient sera**

In order to identify autoantigen modifications by oxidative treatment, sera from patients suffering from SSc (n=72), RP (n=129), PM/Scl (n=52), SLE (n=52), RA (n=4), UCSD (n=3), and MCTD (n=1) were analyzed for autoantibody reactivity towards specific polypeptides on immunoblots containing oxidatively modified cell extract. Figure 2 demonstrates autoantibody reactivity of the patient sera that recognized proteins that were only or preferentially detected in the oxidatively modified material, but not in the control cell extracts. The oxidation-specific protein bands could either be degradation products of proteins with higher molecular weights that are present in the control cell extract, or could represent proteins that are only recognized in the oxidatively modified cell extract through recognition of a neo-epitope. In addition, sera displaying reactivities preferentially or exclusively targeting molecules in the control extract were observed. In these cases, either the oxidative fragments of these proteins were too small to be detected on these western blots, or the oxidative modification had changed the protein in such a way that the patient serum no longer recognized it in the oxidatively modified cell extract. The results, of which an overview is presented in Table 1, were replicated in at least two independent experiments (sera that did not differentially react with the treated and non-treated extract were not included in

Figure 1. Detection of the oxidative fragmentation of the U1-70K protein (70 kDa), leading to the formation of a smear with a molecular weight of 33 to 38 kDa, for Jurkat cells lysed by sonication, centrifuged and clear lysates were incubated with 0.1 mM Fe²⁺ and 1.7 mM ascorbic acid for 30 minutes at room temperature. As a control, cell lysates were incubated for 30 minutes at room temperature in the same buffer but without the addition of Fe²⁺ and ascorbic acid. EDTA was added to a final concentration of 10 mM to stop the reactions, followed by the addition of SDS-PAGE loading buffer and heating for 5 minutes at 95°C. Per lane, cell extract of 5x10⁶ cells were loaded. The control cell extract (−) is run in lane 1, and the oxidatively fragmented cell extract (+) is run in lane 2. Polypeptides are detected with patient serum B16, 20,000-fold diluted, in combination with HRP-labelled rabbit anti-human IgA, IgG, IgM antibodies and ECL. The U1-70K protein is marked with an arrow, and is detected in both lanes. The 33 to 38 oxidation-specific smear is marked with a solid arrow head and a vertical bar. Molecular weight markers are at the left.

Figure 2. Patient sera that differentially react with oxidatively modified and control cell extracts on immunoblot. Patient sera were analyzed for autoantibody reactivity with oxidatively modified (+) and control (−) cell extract. Patient sera were diluted 1,000-fold, and bound human antibodies were detected using HRP-labelled rabbit anti-human IgA, IgG, IgM antibodies and ECL. Patient sera are grouped by disease: SSc patients F14 (lanes 1 and 2), Ks (lanes 3 and 4), Kac (lanes 5 and 6), VhB (lanes 7 and 8), and VG (lanes 9 and 10), RP patients B169 (lanes 11 and 12), F12 (lanes 13 and 14), H111 (lanes 15 and 16), H198 (lanes 17 and 18), and WSS (lanes 19 and 20), and PM/Scl patients Day5 (lanes 21 and 22) and Ven 121 (lanes 23 and 24). Protein bands that are specifically detected in oxidatively modified cell extract are marked with a solid arrow head, and protein bands that are present in the control cell extract, which could no longer be detected in oxidatively modified cell extract, are marked with an open arrow head. Molecular weight markers are at the left of each blot. In lanes 7 and 8, the position of DNA Topoisomerase I (Topo) is indicated on the left.
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RP = Raynaud’s phenomenon; SSc = systemic sclerosis; Th/T o = Th/T o autoantigen (RNase MRP/RNase P); 56K = annexin XI; CENP-B = centromere protein B; PM/Scl = polymyositis/scleroderma overlap syndrome.

Table 1
Overview of patient sera with differential reactivity between oxidatively modified and control cell extracts

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Known autoantibody reactivities</th>
<th>Result immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>F14</td>
<td>SSc</td>
<td>Topo, Th/To, 56K</td>
<td></td>
</tr>
<tr>
<td>Ks</td>
<td>SSc</td>
<td>Topo</td>
<td>-</td>
</tr>
<tr>
<td>Kzc</td>
<td>SSc</td>
<td>-</td>
<td>65, 63 (stronger)</td>
</tr>
<tr>
<td>V84</td>
<td>SSc</td>
<td>Topo</td>
<td>-</td>
</tr>
<tr>
<td>VG</td>
<td>SSc</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B169</td>
<td>RP</td>
<td>-</td>
<td>150, 45</td>
</tr>
<tr>
<td>F12</td>
<td>RP</td>
<td>-</td>
<td>56, 63 (stronger)</td>
</tr>
<tr>
<td>H111</td>
<td>RP</td>
<td>-</td>
<td>150, 45</td>
</tr>
<tr>
<td>H198</td>
<td>RP</td>
<td>CENP-B</td>
<td>80, 30, 22, 20, 18, 15</td>
</tr>
<tr>
<td>W55</td>
<td>RP</td>
<td>Th/To</td>
<td>80, 30, 22, 20, 18, 15</td>
</tr>
<tr>
<td>Day5</td>
<td>PM/Scl</td>
<td>Ro52, La, Jo-1</td>
<td>80, 37, 43, 34, 30, 22</td>
</tr>
<tr>
<td>Ven121</td>
<td>PM/Scl</td>
<td>Ro52, PM/Scl75, PM/Scl100</td>
<td>80, 37, 43, 34, 30, 22</td>
</tr>
</tbody>
</table>

56K = annexin XI; CENP-B = centromere protein B; PM/Scl = polymyositis/scleroderma overlap syndrome; RP = Raynaud’s phenomenon; SSc = systemic sclerosis; Th/To = Th/To autoantigen (RNase MRP/RNase P); Topo = DNA topoisomerase I.

The table). Differential reactivity was observed with five SSc patients, five RP patients, and two PM/Scl patients. It should be noted that such reactivities were not seen in any of the 15 control sera.

SSc patient F14 (Figure 2, lanes 1 and 2) reacted with proteins of approximately 200, 170, 100 and 55 kDa in both cell extracts, and with three additional proteins of around 150, 140 and 130 kDa in the oxidatively modified extract. In case these three oxidation-specific proteins result from oxidative fragmentation, it is unclear whether they derive from the 200 kDa or from the 170 kDa proteins, since no reduction in signals from these proteins in the oxidatively modified cell extract was observed, as would be expected upon protein degradation. However, detection of these oxidation-specific proteins could also result from recognition of neo-epitopes that are only present on the oxidatively modified protein. In this case, this patient serum would contain autoantibodies to the oxidatively modified form of these proteins with no detectable autoantibodies to their non-modified counterparts. Serum of SSc patient Ks (Figure 2, lanes 3 and 4) reacted with a protein smear of 35 to 37 kDa, which was not detected in the oxidatively modified cell extract. SSc patient serum Kzc (Figure 2, lanes 5 and 6) demonstrated unusual differential reactivities with oxidatively modified and control cell extracts, since the oxidation-specific target protein (36 kDa) migrated slower in SDS-PAGE gels than the putatively corresponding protein in the control cell extract (35 kDa). Serum of SSc patient V84 (Figure 2, lanes 7 and 8) reacted with proteins of approximately 150 and 100 kDa in the control and the oxidatively modified cell extracts. The 100 kDa protein is thought to represent DNA topoisomerase I (Topo), since V84 tested positive for autoantibodies directed to this 100 kDa protein in routine laboratory assays. V84 reacted with proteins of 95, 65 and 45 kDa that were specifically present in the oxidatively modified cell extract. The 95 kDa protein might result from oxidative fragmentation of Topo, as described previously (25), although we were unable to detect this polypeptide with other anti-Topo positive patient sera. It is unclear whether the proteins of 65 and 45 kDa derive from the 150 or the 100 kDa protein that is present in the control cell extract, since autoantibody reactivity with neither of these proteins were reduced in the oxidatively modified extract. Autoantibodies in the serum of SSc patient VG (Figure 2, lanes 9 and 10) recognized proteins of 80 and 17 kDa in control cell extract. The protein of 17 kDa was no longer detected in the oxidatively modified cell extract.

RP patient serum B169 (Figure 2, lanes 11 and 12) recognized proteins of 150, 45 and 35 kDa in the control cell extract, the latter of which was not detected anymore in the oxidatively modified cell extract. Furthermore, proteins of approximately 63 and 65 kDa were more strongly recognized in oxidatively modified cell extract compared to the control cell extract. Autoantibodies in serum of RP patient F12 (Figure 2, lanes 13 and 14) reacted with proteins of 100, 94, 80 and 70 kDa in the control cell extract, but the 80 and 70 kDa proteins were no longer detected in the oxidatively modified extract. RP patient H111 (Figure 2, lanes 15 and 16) reacted with proteins of approximately 100, 44, 32, 25, 24, and 21 kDa in the control cell extract. In the oxidatively modified cell extract all proteins were detected, except for the 21 kDa protein. RP patient serum H198 (Figure 2, lanes 17 and 18) reacted with proteins of 80, 30, 22, 20, 18 and 15 kDa in the control cell extract. In the oxidatively modified extract an additional protein of 29 kDa was recognized as well. RP patient serum W55 (Figure 2, lanes 19 and 20) demonstrated autoantibody reactivity to proteins of approximately 120, 55, and 35 kDa in the control cell extract. In the oxidatively modified extract, the 55 kDa protein was no longer detected, whereas a polypeptide of 22 kDa was additionally recognized. This finding might indicate that the 55 kDa protein is fragmented to the 22 kDa protein. The 22 kDa oxidation-specific protein seemed to be recognized somewhat more efficiently than the 55 kDa protein, which might result from autoantibodies to neo-epitopes on the oxidation-specific fragment.

Serum of PM/Scl patient Day5 (Figure 2, lanes 21 and 22) reacted weakly with proteins of 80, 34, 30, and 22 kDa, and strongly with protein(s) of 37-43 kDa in both the control and the oxidatively modified cell extract. In the oxidatively modified cell extract, Day5 reacted with an additional protein of approximately 25 kDa. PM/Scl patient serum Ven121 (Figure 2, lanes 23 and 24) reacted with 80, 70 kDa and 35 kDa proteins in control cell extract. In the oxidatively modified cell extract the 35 kDa protein was no longer detected after oxidation, whereas a protein of 37 kDa was additionally present. Possibly, the 37 kDa protein could represent a modified form of the 35 kDa proteins.
protein in control cell extract, indicating that its molecular weight was slightly increased by other modifications than cleavage. It is also feasible that the 35 kDa protein was degraded to smaller fragments that were not detected on this western blot, and that the 37 kDa protein results from fragmentation of the 80 or the 70 kDa proteins.

We also analyzed a set of monoclonal antibodies (mAb) directed to well-characterized autoantigens, including anti-La mAb SW5, anti-Ro52 mAb 4C6, anti-Sm mAb Y12, anti-Sm-B/B’ mAb ANA125, and anti-U1A/U2B’ mAb 9A9, and a polyclonal rabbit antibody directed to poly-(ADP-ribose) polymerase (PARP). None of these antibodies demonstrated differential reactivities (data not shown), indicating that their target autoantigens are not detectably altered in the oxidatively modified cell extract.

Autoantibody targets generated by oxidative modification are not generated during cell death

Two patient sera with strong reactivity against oxidation-specific polypeptides, V84 and F14, were analyzed on immunoblots containing apoptotic, necrotic and oxidatively modified cell extracts. Apoptosis was induced by Fas ligand (29) or anisomycin (18) for 8 hours at 37°C. After 8 hours, the 70 kDa U1-70K protein was completely cleaved to the apoptosis-specific 40 kDa fragment (data not shown), which was detected by western blotting using a recombinant anti-U1-70K monoclonal antibody (17). The U1-70K cleavage is regarded as a biochemical marker for apoptosis (30). Necrosis was induced by incubation with 200 μM mercuric acetate for 7 hours at 37°C. After 8 hours, approximately 80% of the cells were necrotic, as determined by a trypan blue assay (data not shown). Figure 3 shows the autoantibody reactivities of patient sera V84 and F14 with antigens present in apoptotic, necrotic and oxidative cell extracts. The oxidation-specific polypeptides of 95, 65 and 45 kDa recognized by V84 were clearly recognized in the oxidatively modified cell extract, but not in the control, the apoptotic and the necrotic cell extracts. A fragment of approximately 70 kDa was specifically detected in apoptotic cell extracts, both induced by the drug anisomycin and by Fas ligand, and was believed to result from cleavage of Topo (10). Also the oxidation-specific polypeptides of around 150, 140 and 130 kDa recognized by F14 were clearly detected in the lane with oxidatively modified cell extract. These protein bands were neither present in the control cell extract, nor in the lanes containing apoptotic or necrotic cell extract. In addition, patient serum F14 recognized a polypeptide of approximately 70 kDa in the apoptotic material, both after induction of apoptosis by anisomycin and by Fas ligand. This protein fragment was believed to derive from apoptotic cleavage of Topo, which is supported by the fact that patient F14 was known to possess autoreactivity with this autoantigen (see Table 1). No differences were detected between the necrotic and the control cell extracts. These results demonstrate that the neo-epitopes on the polypeptides recognized by these sera after oxidative modification are unique for this process, and are not created during apoptosis and necrosis.

Discussion

To date, experimental data on oxidative modifications of autoantigens are sparse. Here we report novel patterns of oxidatively modified proteins that are recognized by autoantibodies in sera from patients suffering from scleroderma, or from scleroderma-associated conditions such as Raynaud’s phenomenon and polymyositis/scleroderma overlap syndrome. The fact that the oxidative fragments are not generated during apoptosis and necrosis indicates that neo-epitopes on oxidatively modified proteins exist and that they are different from apoptotic and necrotic neo-epitopes on these autoantigens. Since we did not find antibody activities to oxidatively modified protein (fragments) in control sera, our results support the idea that modifications during oxidative stress are linked to autoantibody formation in scleroderma and scleroderma-related conditions.

The appearance of specific autoantibody reactivities with the oxidatively modified material may be explained in two ways. First, the electrophoretic mobility of proteins that are also targeted in the non-modified extract might be changed, e.g. by fragmentation, which can be expected to occur in the presence of radicals (25,27). Second, the modification of polypeptides may lead to the generation of neo-epitopes, which are targeted by the antibodies in these sera. Finally, also a combination of these phenomena may occur when the modification that affects the electrophoretic mobility also generates a neo-epitope. The finding that autoantibodies in several patient sera (for example F14 and V84) reacted
strongly with polypeptides in an oxidative modification-dependent manner, which did not coincide with an apparent reduction in other autoantibody reactivities of these sera. This suggests that the generation of neo-epitopes is the most important process. Clearly, our study does not provide insight into the molecular identity of the polypeptides that contain neo-epitopes created during the oxidative treatment. Immunoaffinity purifications of the proteins from oxidatively modified cell extracts will be required to shed more light on this. Also further studies are necessary to elucidate the exact nature of these neo-epitopes.

When the polypeptides that are specifically targeted by the autoantibodies in the oxidatively modified extract are related to autoantibody targets in the non-modified extract, part of the reactivity may be due to antibodies recognizing also the non-modified proteins. Competition experiments, in which serum is pre-incubated with control cell extract in order to compete for binding of autoantibodies to common epitopes, might provide more insight into this issue.

Although several distinct patterns of oxidative modification could be identified, the fragmentation pattern described for the scleroderma autoantigen DNA topoisomerase I (from a 100 kDa intact protein to 95 kDa oxidative fragment), could only be detected with one patient serum (V84), and not with at least 12 other sera with strong anti-Topo reactivity. This observation suggests that the oxidation-specific protein of 95 kDa detected by serum V84 is probably not generated through fragmentation of DNA topoisomerase I, but corresponds to another protein instead. The fact that Topo fragmentation, as described by Casciola-Rosen et al. (25), was not detected in our experiments might be due to the difference in cell lines used: Topo fragmentation was previously demonstrated in HeLa cells (25,27), whereas Jurkat cells were used in the study presented here. Tissue- or cell-type specific modifications have indeed been described before. For example, Ulanet et al. published that the scleroderma autoantigen B23 was very efficiently cleaved in differentiated vascular smooth muscle cells upon incubation of the cells with granules containing granzyme B, whereas it was not cleaved in undifferentiated vascular smooth muscle cells and fibroblasts that were treated in the same way (31). We used Jurkat cells in this study, because apoptotic pathways are well-characterized in these cells, and because Jurkat cells have been extensively used for characterization of autoantigen modifications during apoptosis and necrosis. This would also allow a comparison of oxidative modifications with apoptotic and necrotic modifications. On the other hand, it might be important to study such modifications in cells that are exposed to oxidative stress in vivo. In the case of SSc and RP patients, such processes may occur in the endothelial cells that form the inner lining of blood vessels, and in fibroblasts, which are present in the underlying connective tissue.

As stated above, besides radical-mediated polypeptide fragmentation other modifications may occur under oxidative stress conditions, in particular modifications of amino acid side chains. This possibility is indeed supported by the finding of serum reactivities with oxidation-specific proteins that migrate slower in polyacrylamide gels compared to the putative corresponding protein in control cell extracts.

Differential reactivity with oxidized proteins (or fragments thereof) was observed in a relatively low number of patient sera. Out of a total of 225 sera only 12 sera were selected based upon their differential reactivity patterns. This raises the question whether the immune response against the modified proteins is relevant for the pathophysiology of the disease. In addition, it is puzzling why the same reactivities were not observed with different patient sera, in particular within one disease group. An important point to consider in this regard is that the serum samples used in general were withdrawn from the patients at relatively late stages of the disease process. The reactivity to the modified antigens is likely to be associated with the (very) early phases of the disease and it is known that such reactivities may become less prominent during further development of the disease. Furthermore, it has been shown that other processes of cell death, or other phenomena, may play a role as well. Differences between individual patients may cause differences in reactivity patterns. For example, a set of scleroderma autoantigens has been described to be uniquely cleaved by granzyme B, and a 60 kDa fragment, generated by granzyme B cleavage (and presumably derived from centromere protein C) correlated with ischemic digital loss in scleroderma patients (32). These findings implicate CTL-induced cell death as the underlying mechanism in breaking tolerance to self-antigens in these specific patients. The molecular characterization of the antigens and epitopes that are responsible for the differential reactivities observed will, besides improving the sensitivity and specificity of the assay, facilitate a more systematic and more widespread analysis of the occurrence of these autoreactivities in autoimmune sera. Because only low numbers of SLE, RA, UCTD and MCTD sera were analysed in parallel in our study and only low frequencies of differentially reactive sera were observed for the other patient groups, the absence of such sera in the SLE, RA, UCTD and MCTD groups does not allow conclusions on the recognition of oxidatively modified antigens by these patients.

In conclusion, we have shown that sera from patients with scleroderma or scleroderma-related conditions contain autoantibodies to antigens that are uniquely generated in cell extracts after treatment with oxidative reagents. Our results thus support the concept that modifications generated during oxidative stress are linked to autoantibody formation in these diseases.

Materials and Methods

Patient sera

Patient sera were obtained from the serum collection of the Department of Biochemistry, Radboud University Nijmegen (The Netherlands). These patients had been seen at the Department of Rheumatology of the University Medical Centre Nijmegen or the St. Maartenskliniek Nijmegen (The Netherlands), and had been classified in accordance with standard criteria for each disease. Patient sera were present from SSc (n=72), RP (n=129), PM/ScI...
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(n=24), SLE (n=5), RA (n=4), UCTD (n=5), and MCTD (n=1). Informed consent was obtained from all participants, and sera were stored at -70°C until use.

Cell lines
Jurkat (human T cell leukemia) suspension cells were grown in RPMI 1640 medium (Gibco-BRL), supplemented with 1 mM sodium pyruvate, 1 mM penicillin, 1 mM streptomycin and 10% heat-inactivated foetal calf serum (Gibco-BRL), in a humidified 37°C incubator containing 5% CO₂. Cells were maintained at a concentration of 10⁶ cells/ml.

Oxidative fragmentation of cell extracts
Oxidative fragmentation was carried out as described previously for HeLa cells (27), with minor changes. Jurkat suspension cells were harvested by centrifugation at 800 x g for 5 minutes, and washed twice with KRB wash buffer (200 mM HEPES, pH 7.4, 127 mM NaCl, 5.5 mM KCl, 10 mM dextrose, 1 mM CaCl₂, and 2 mM MgSO₄). Cells were resuspended in lysis buffer 1 (25 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.05% NP-40, 10% glycerol, and EDTA-free Complete™ protease inhibitor cocktail (Roche)). Cells were lysed by sonication on ice, and centrifuged at 12,000 x g and 4°C for 20 minutes. Clear lysates were used immediately or stored at -70°C.

For induction of necrosis, cells were harvested and washed with PBS. Cells were resuspended at a concentration of 1x10⁶ cells/ml in incubation buffer (50 mM HEPES, pH 7.6, 100 mM NaCl, 0.4 mM Ca(NO₃)₂, 5.4 mM KCl, 0.4 mM MgSO₄, 6.7 mM NaH₂PO₄, and 1 mM dithioerythritol), which resembles the composition of RPMI medium. Cells were incubated in the presence of 200 µM Hg(CH₂COO)₂ for seven hours at 37°C. The cell suspension was sonicated on ice in the presence of Complete™ protease inhibitor cocktail (Roche), and centrifuged at 4,000 x g and 4°C for 15 minutes. The pellet, containing amongst others proteins that precipitated during necrosis, was resuspended in the original volume of two-fold concentrated SDS-PAGE loading buffer, and pooled with the supernatant, which contained soluble protein (fragments) that had leaked out of the cells. Samples were heated for 5 minutes at 95°C prior to gel electrophoresis.

SDS-PAGE and immunoblotting
Proteins from cell extracts were separated by SDS-PAGE, and were subsequently transferred to nitrocellulose membranes (Schleicher&Schuell) by semi-dry electroblotting. Protein transfer was checked by Ponceau S staining. Per lane proteins from approximately 5x10⁴ cells were loaded. All incubation steps were carried out at room temperature on a shaking table. The blots were blocked with 5% non-fat dried milk powder in PBS, containing 0.05% Tween-20 (MPBST), for one hour. Patient sera were incubated with the blot membrane at a 1,000-20,000-fold dilution in MPBST, for one hour. Bound antibodies were detected with horseradish peroxidase (HRP)-labelled goat-anti-human IgA,IgG,IgM antibodies (DAKO), 1,000-fold diluted in MPBST, followed by enhanced chemiluminescence (ECL) detection. After antibody incubations, membranes were washed extensively with MPBST to reduce background signals.

References


Chapter 9

General discussion
Since its initial discovery in 1985, the antibody phage display technology has gone through major developments, and a number of sophisticated applications have been designed. The first part of this chapter intends to discuss our antibody phage display results and to put them in perspective of other developments in the field of antibody phage display technology. The data presented in the second part of this thesis support the hypothesis that cell death-specific epitopes are involved in breaking tolerance to self-antigens. The implications of these results are discussed in relation to recent findings in other studies.

Part 1: Antibody phage display technology

Formats and applications of recombinant antibody fragments

Since single chain variable fragments (scFvs), and Fabbs, are easily cloned and expressed in bacteria and mammalian cells, it is relatively simple to fuse them with various peptide tags and proteins. If the activity of the resulting recombinant antibody is unaltered and the fusion protein can be functionally expressed, the possible applications of such constructs are almost unlimited.

In general, therapeutic antibodies, directed to specific cell types or cells in a certain pathological condition (for example tumour cells), are labelled with specific chemicals, or conjugated or fused to proteins that affect (e.g. kill) the target cell. Such fusion proteins are for example cytokines (1), enzymes that can convert pro-drugs (with no cytotoxicity) into active drugs (with cytotoxicity) in antibody-directed enzyme pro-drug therapy (2,3), Fas ligand (4), granzyme B (5), and tumour necrosis factor-alpha (TNF-alpha) (6).

For research purposes, many tags are available with a variety of applications. The vesicular stomatitis virus glycoprotein (VSV-G) (7), c-myc- (8,9), and Flag-tags (10) are examples of small peptide sequences that are widely used for this purpose. In combination with anti-VSV-G, anti-c-myc and anti-Flag antibodies, tagged scFvs are easily detected in various immunological assays.

Examples of tags that can be used for purification of scFvs (and proteins in general) are the hexahistidine-tag (11), maltose binding protein (12), the streptavidin-binding-tag (13), glutathione-S-transferase (14), and different domains of the Staphylococcus aureus protein A (15). Furthermore, fusion of recombinant antibodies to such tags allows immunoaffinity purification of their antigens from complex protein mixtures. For example, in Chapter 4, scFvs directed to unknown targets were C-terminally fused to the hexahistidine-tag for immobilization on Ni²⁺-NTA agarose beads. After incubation with cell extract, the bound antigens were eluted from the beads, separated on polyacrylamide gels, and identified by mass spectrometry.
For the use of scFvs in localization studies, peptide tags like the VSV-G-tag can be used, but several other approaches have been described as well. For example, scFvs have been fused to the green fluorescent protein (GFP) (16), and scFvs can equally well be fused to red, yellow and blue fluorescent proteins (17,18) in order to perform double- or triple-staining studies. Another method for direct localization of target antigens is fusion of scFvs to alkaline phosphatase (19). Similarly, it should be possible to fuse scFvs to horseradish peroxidase, although such a format has not been described so far.

The approach described in Chapter 3 is an attractive additional format for localization studies. Here, scFvs were fused to constant domains from immunoglobulin light chains from different species (human, mouse and chicken), and detected with commercially available, fluorescently labelled secondary antibodies directed to human, mouse and chicken immunoglobulins. The main advantage of this method compared to the approaches described above is that a large number of combinations are possible (one is not dependent on the animal the antibody is derived from), since the secondary antibodies with different fluorescent labels are readily available. The diversity of combinations that is generated by using the light chain constant domains from human, mouse and chicken, can be further expanded by fusing scFvs to light chain constant domains from other species. In such systems rabbit and goat are attractive alternatives, since secondary antibody conjugates to rabbit and goat immunoglobulins are commercially available. Furthermore, next to their use in localization studies, the constant domains can also be used for detection of the scFvs in ELISA or immunoblotting. A disadvantage of recombinant antibodies is that they are, in general, less stable compared to complete immunoglobulins like monoclonal hybridoma antibodies. However, the stability can be improved by, for example, mutagenesis (20) or loop grafting (21). One can also use single domain antibodies, which can be isolated from cameld or shark libraries, and which have proven to be highly stable (22).

Chapter 3 also demonstrated that fusion of scFvs with constant light chain domains leads to partial scFv dimerization, although the level of dimerization is not as high as with Jun domains, which are leucine zipper domains that can mediate dimer formation (23,24). The fusion of constant light chain domains may also increase scFv avidity (24), although this still has to be confirmed by experimental data.

Antibody phage display systems

For ethical and economic reasons, it is valuable to reduce the number of test animals needed in antibody phage display. This can be achieved (1) by using naïve or synthetic libraries (no animals needed), or (2) by preparing immune libraries from animals that have been simultaneously immunized with multiple antigens (fewer animals needed). Although high affinity recombinant antibodies can be isolated from naïve or synthetic libraries, this is only possible if the libraries are very large (i.e. a complexity of 10^11 or higher) (25). The preparation of such large antibody libraries is difficult and laborious (see review by (26)), and nowadays such complex libraries have been made only by a few companies, such as Cambridge Antibody Technology (library with more than 10^10 different antibodies), MorphoSys (HuCAL library, which contains more than 10^10 different antibodies), and Dyax (libraries with more than 10^10 different antibodies), and thus protected from use by others. Alternatively, moderate affinities of isolated antibodies can be increased by mutagenesis, although this is a time-consuming step that has to be carried out for each individual antibody (25). If an antibody with relatively high affinity is not readily isolated from a synthetic library, or if a range of antibodies to one particular antigen is needed, immunization and preparation of an immune library might be a better approach (25). This option also offers a suitable alternative when high affinity antibodies are needed. In Chapter 2, we demonstrated that antibodies could be isolated from chickens that were simultaneously immunized with mixtures of antigens. A similar approach had also been used in rabbits (27), but chicken offers two additional advantages. First, chickens are, compared to rabbits, phylogenetically more distant from human, and thus more likely to evoke an immune response to conserved epitopes on human molecules. Second, although preparation of libraries from rabbits already involves less primer sets compared to the preparation of libraries from mice or humans, chicken libraries need only two primer sets, which significantly decreases the time for library preparation. In conclusion, multiple antigen immunization reduces the number of animals needed, where chickens are an attractive source for antibody generation.

Target discovery by antibody phage display

As described in Chapter 1, several methods have been designed for the identification of proteome-specific targets. The great advantage of such methods is that at the same time that the targets are discovered, monoclonal antibodies directed to these targets are generated, and both the antigen and the antibody are potential therapeutic or diagnostic tools. The novel subtractive approach described in Chapter 4 can be regarded as a combination of three existing approaches: selection in solution on biotinylated antigens (28,29), subtractive selection, and selection on antigens immobilized on blotting membranes, (30-33). After selection to biotinylated antigens in solution, monoclonal phages had to be individually analyzed for binding reactivity on immunoblot strips containing normal and apoptotic cell extract in a rather laborious screening procedure. Therefore, the last part of the selection procedure, the enrichment step on blot, was included to reduce the number of phages that needed to be analyzed. The combination of subtractive selection, selection in solution and selection on blotting membranes might result in recombinant antibodies that recognize an epitope that is available in solution as well as on blot, and such antibodies are thus expected to be applicable in a wide range of assays. A disadvantage might be that some phages that do bind differential epitopes during selection in solution might not be selected in the second part of the procedure, for example when they are directed to a conformational epitope that is not available anymore on the blot after the blotting procedure.
The extra enrichment step on immunoblot is especially suitable when no robotic equipment is available. When robotic equipment can be used, it seems preferable to perform one selection round on (biotinylated) antigens in cell extracts, which keeps the diversity relatively high compared to performing more selection rounds, and subsequently screen as many monoclonal phages as possible. Nevertheless, it will still be difficult to analyze many monoclonal phages individually on blotting strips, but a capture ELISA with both extracts could be developed. A drawback of the blotting approach is that differences in molecular weight, for example those of apoptosis-specific epitopes generated after specific cleavage of certain proteins, might be missed in this way.

Although the method described in Chapter 4 is designed for the identification of intracellular targets, it can be used for extracellular and cell surface proteins as well. In this case, cell surface proteins are isolated and, after biotinylation, used for the selection. Furthermore, the method should be applicable using non-biased naïve of synthetic libraries, although chances of isolating a specific target are believed to be higher when immune libraries with higher affinity reactivities towards this protein. It would be valuable to determine whether autoantibodies in patient sera to hnRNP C react stronger with the apoptotic variant of these proteins, as was demonstrated for the U1-70K protein (Chapter 6). In Chapter 7, we showed that PSF/p54\textsuperscript{ab} is a minor autoantigen as well. Reactivity to recombinant p54\textsuperscript{ab}, though with low titres, was found in one patient serum used for the screening of the libraries (Chapter 4), and in two patients suffering from PM/ScI overlap syndrome. In addition, the experiments described in Chapter 7 demonstrate that p54\textsuperscript{ab}/PSF and hnRNP C translocate from their nuclear localization in normal HeLa cells to apoptotic bodies or apoptotic blebs at the cell surface. This phenomenon has been described for many autoantigens, including U1-70K (36,37). Since proteins are believed to be more easily exposed to the immune system when they are present in apoptotic blebs, the presence of p54\textsuperscript{ab}/PSF and hnRNP C in such apoptotic structures supports (though not proves) the postulation that these proteins can trigger an autoimmune reaction.

After the selection steps, three monoclonal phages were chosen that strongly react with proteins that are differentially present in normal and apoptotic cell extracts. These three monoclonal phages were used to further set-up the identification procedure, which included isolation of the target antigen from cell extracts by immunoprecipitation, and identification of the target by mass spectrometry. Of course, it would be of much interest to isolate other monoclonal phages from the pool of phages that eluted from the individual spots, since these might target other (disease) markers of interest. It would also be valuable to study other cell death-specific epitopes on autoantigens, for example those created during necrosis or oxidative damage, by a similar approach.

**Concluding remarks part 1**

In short, the methods developed and described in this thesis extend the range of current methods using antibody phage display and recombinant antibodies, and provide new means to detect specific (apoptotic) autoantigens in autoimmune diseases. The differential proteomic selection method may be more widely applicable in the identification of antigens that are uniquely expressed in one proteome but not in another.

**Part 2: The role of cell death-specific epitopes in breaking tolerance to self-antigens**

**How can tolerance to self-antigens be broken?**

One model for breaking immunological tolerance to ‘self-antigens that is gaining a lot of attention is the molecular mimicry model. In this model, which has been mainly linked to SLE, the production of autoantibodies in patients is believed to be initiated through cross-reactivity with epitopes on bacteria or viruses. Autoantibodies directed to the SME autoantigens Sm-B/B’, Sm-D and R060 have been found to cross-react with the Epstein-Barr virus nuclear antigen-1 (EBNA-1) (see reviews by (38-40)), and this virus has been found significantly more frequently in SLE patients as compared to controls (41,42).

Recently, McClain et al. identified an epitope on R060, which was recognized by serum samples from SLE patients before they were diagnosed (43). It was demonstrated that autoantibodies to this early autoepitope cross-reacted with a peptide contained in EBNA-1. Immunization of rabbits with peptides based on either the initial epitope on R060 or the cross-reactive EBNA-1 epitope, resulted in autoantibodies to multiple epitopes on R060, with subsequent epitope spreading to epitopes on Sm-B’, RNP and dsDNA. Furthermore, the immunized rabbits developed some symptoms that are also found in SLE patients. A similar cross-reactivity has also been suggested to exist between R060, which is next to SLE also associated with Sjögren’s syndrome, and Coxsackie virus 2B protein (44). This finding is
in accordance with the detection of Coxsackie virus RNA in biopsies of salivary glands from patients suffering from Sjögren’s syndrome (39). Also in the molecular mimicry model, the genetic background is believed to contribute to an individual’s susceptibility to develop an autoimmune response, since many people have been infected with Epstein-Barr virus (42), though only a small percentage develops SLE.

Another model involves anti-idiotypic antibodies. Idiotypes are the antigenic constitutions of the variable region of an antibody, and they are recognized by anti-idiotypic antibodies, which are known to occur in patients suffering from autoimmune diseases (see review by (45)). It is believed that antibodies carrying idiotype can induce production of anti-idiotypic antibodies, which subsequently may trigger the production of anti-anti-idiotypic antibodies. Consequently, a network is created of interacting antibodies in which the idiotypic determinants of each antibody are complemented by those of another. Recently, Pendergraft et al. launched a theory, which they named the autoantigen complementarity theory (46). In this theory it is hypothesized that a (microbial) protein whose amino acid sequence is (in part) structurally the complement of that of a self-antigen will generate an immune response. The idiotypic antibody that is produced elicits a second immune response to the first antibody, resulting in an anti-idiotypic antibody. Due to the complementarity of the initial triggering antigen, the idiotypic antibody mimics the complementary autoantigen, and as a consequence the anti-idiotypic antibody is directed to the autoantigen. By performing homology searches of complementary protein sequences, these authors found many microbial and fungal proteins with complementing abilities, supporting the idea that invading microorganisms indeed can deliver the initiating immunogen.

In a third model it is hypothesized that modified self-antigens are released from dying cells and that these modifications can break immunological tolerance to these self-antigens. This model will be discussed extensively in the following paragraphs.

Do autoantigens display cell death-specific epitopes?

More and more experimental data support the hypothesis that neo-epitopes, resulting from post-translational modifications of autoantigens during cell death, can trigger an autoimmune reaction. For example, peptidylarginine can be converted into peptidylcitrulline, which is catalyzed by peptidylarginine deiminases, enzymes that are known to be particularly active in dying cells. Anti-citrullinated protein antibodies (ACPA) are tightly associated with rheumatoid arthritis (RA) (see Table 1). Recently, Lundberg et al. demonstrated in a rat model that citrullinated proteins can break tolerance to self-antigens (47). Immunization of rats with citrullinated rat serum albumin (RSA) led to the production of antibodies to citrullinated (and non-citrullinated) RSA, whereas immunization with non-modified RSA did not. As described in Chapter 5, important additional support for the hypothesis that neo-epitopes play a role in breaking tolerance to self-antigens is the finding that the U1-70K protein displays apoptosis-specific epitopes, and that such epitopes are recognized by specific autoantibodies in anti-U1-70K patient sera (48,49). The experiments described in Chapter 6, which demonstrate that the majority of anti-U1 small nuclear ribonucleoprotein (snRNP) sera preferentially recognize apoptotically cleaved U1-70K as compared to intact U1-70K on immunoblot, confirm these data. Moreover, the immunoprecipitation experiments shown in Chapter 4 demonstrate that apoptosis-specific epitopes on the U1-70K protein are also recognized by the recombinant antibody obtained via the subtractive phage display approach described in this chapter, as well as by recombinant antibodies isolated from SLE-derived antibody phage display libraries as described in a previous study by Degen et al. (50). The additional finding that early patient sera are relatively enriched for autoantibodies directed to the apoptotic fragment of U1-70K (Chapter 6) further supports the assumption that epitopes that are specifically present on apoptotic U1-70K are involved in breaking tolerance to U1-70K and the U1 snRNP complex.

The La protein, a protein binding to many different RNAs, is an important autoantigen associated with Sjögren’s syndrome (see review by (51)). Recently, a neo-epitope was found to

### Table 1: Association of autoantibody reactivities and HLA haplotypes with systemic autoimmune diseases

<table>
<thead>
<tr>
<th>Autoimmune disorder</th>
<th>Examples of serological markers</th>
<th>HLA haplotype associated with disease or autoantibody reactivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>anti-dsDNA, anti-Sm</td>
<td>DRB1<em>0301,0801,1501; DQB1</em>0201,0402,0602</td>
<td>(109)</td>
</tr>
<tr>
<td>SSc (diffuse)</td>
<td>anti-DNA Topoisomerase I</td>
<td>DR5/11 (DRB1<em>1101,1104; DQB1</em>0301; DRB1*1301)</td>
<td>(110)</td>
</tr>
<tr>
<td>SSc (limited)</td>
<td>anti-centromere proteins</td>
<td>DR1 (DRB1<em>0101; DQB1</em>0501)</td>
<td>(110)</td>
</tr>
<tr>
<td>RA</td>
<td>anti-CCP (ACPA)</td>
<td>DR4 (DRB1*0401)</td>
<td>(92)</td>
</tr>
<tr>
<td>PM/DM</td>
<td>anti-Jo-1</td>
<td>A<em>01; B</em>08; Cw<em>0701; DRB1</em>0301; DQA1*0501</td>
<td>(94)</td>
</tr>
<tr>
<td>PM/DM</td>
<td>anti-M2</td>
<td>DRB1<em>0701; DQA1</em>0201</td>
<td>(94)</td>
</tr>
<tr>
<td>PM/Sc</td>
<td>anti-PM/Scl-100; anti-PM/Scl-75</td>
<td>DR13 (DRB1<em>0301; DQB1</em>0501)</td>
<td>(110)</td>
</tr>
<tr>
<td>MCTD</td>
<td>anti-U1 snRNP (anti-RNP)</td>
<td>DR4 (DRB1<em>04; DQB1</em>0302)</td>
<td>(110)</td>
</tr>
</tbody>
</table>

ACPA = anti-citrullinated protein/peptide antibodies; CCP = cyclic citrullinated peptide; dsDNA = double stranded DNA; Jo-1 = histidyl tRNA synthetase; MCTD = mixed connective tissue disease (SLE overlap disease); PM/DM = polymyositis/dermatomyositis; PM/Sc = polymyositis/scleroderma overlap syndrome; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus; SSc = systemic sclerosis (scleroderma); U1 snRNP = U1 small nuclear ribonucleoprotein

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be created on the La protein upon cleavage by granzyme B. Autoantibodies reactive with this cell death-specific epitope were detected in a number of sera from patients with Sjögren’s syndrome (52), indicating that this epitope might be involved in breaking tolerance to La in these patients. Furthermore, Schachna et al. demonstrated a preferential recognition of protein fragments derived from systemic sclerosis (SSc, also referred to as scleroderma) autoantigens after treatment with granzyme B, which suggests that granzyme B cleavage also might create novel epitopes on SSc autoantigens (53).

In Chapter 8, it was demonstrated that autoantibodies in sera from patients suffering from SSc, Raynaud’s phenomenon (RP) and polymyositis/scleroderma overlap syndrome (PM/Scl) recognize proteins that are modified upon oxidative damage. This finding further supports the hypothesis that autoantibody production in these patients may be triggered by a neo-epitope, created on these antigens during tissue damage by reactive oxygen species. Nevertheless, more experimental data are needed to demonstrate the existence of such neo-epitopes and the presence of autoantibodies directed to them in patient sera.

In conclusion, to date a small though increasing number of autoantigens has been described to display neo-epitopes that are needed to demonstrate the existence of such epitopes. Can the type of cell death predict disease outcome?

Table 1 provides examples of autoantibodies that are regarded as markers for disease, because of their tight association with a certain disease, as for instance autoantibodies to cyclic citrullinated peptides (CCP) (99% specificity for RA (54)) or DNA topoisomerase I (highly specific for SSc, see review by (55)). The question why autoantibodies arise in autoimmune patients, and why certain autoantibody specificities are associated with particular autoimmune diseases remains intriguing and as yet unanswered. It can be assumed that several factors play a role; the most important of these will be addressed in the following sections.

Protein modifications may depend on cell death processes

Cell death is part of the normal development and life cycles of cells, and the most common (and most extensively studied) types of cell death are apoptosis and necrosis. For an overview of apoptotic and necrotic processes and pathways, the reader is referred to a selection of reviews (56-59). In short, the main phenotypical differences between the two major forms of cell death are cell membrane blebbing, chromatin condensation, cell shrinkage, and DNA fragmentation during apoptosis, whereas necrosis is characterized by cell swelling, loss of cell membrane integrity, and disruption of the cell. In contrast to the situation during apoptosis, the content of necrotic cells is released into the cell’s environment, resulting in damage to the surrounding cells and a local inflammatory response.

The typical molecular responses occurring in the various types of cell death differ as well. Apoptosis can be divided into an intrinsic pathway (characterized by release of cytochrome C from the mitochondria upon induction by a variety of stimuli) and an extrinsic pathway (induced when death receptors, such as Fas or the TNF receptor, are activated through binding with their ligands). In both apoptotic pathways, caspases (cysteine proteases that cleave after aspartic acid) are key players (60,61). After activation, these caspases cleave substrate proteins (62), and thereby activate, either directly or indirectly, enzymes that subsequently modify other molecules in the dying cell. Examples of such modifications are (de)phosphorylation, transglutamination and citrullination of proteins (63), cleavage of chromosomal DNA into nucleosomal units (64), and cleavage of RNA (see review by (65)).

In addition, cells can be stimulated to go into apoptosis by cytotoxic T lymphocytes (CTLs), which serve to eliminate virus-infected cells and tumour cells (see reviews by (66,67)). After recognition of a target cell, cytotoxic granules containing perforin and granzymes are released from the CTLs. The granzymes enter the target cell through pores that are formed by the perforin molecules, and activate caspase-dependent and -independent apoptotic pathways (see reviews by (68,69)).

Granzyme B is a serine protease that is able to cleave proteins at the same sites as caspases, i.e. at aspartic acid residues (see review by (70)), but it can also cleave proteins at other sites which are not susceptible to caspase cleavage (71).

For necrosis, only cleavage has been described as a possible source of protein modifications (72). Here, cathepsins and calpains are described as the main cleaving enzymes (73,74), although these enzymes can also be activated during apoptosis (75,76). An additional source of protein modification is oxidative fragmentation by radicals, which has been linked to SSc autoantigens (77,78). Such radicals are believed to be formed during ischemia-reperfusion, a process that is known to occur in the skin of patients with SSc and Raynaud’s phenomenon (see review by (79)).

As summarized in Table 2, the various proteolytic enzymes (and radicals) that are activated during the different processes of cell death cleave their target proteins at different recognition motifs, which implies that they create different neo-epitopes on these proteins. An example of a protein that is differentially cleaved during the different processes of cell death is the U1-70K protein. U1-70K is cleaved by caspase-3 resulting in the appearance of a 40 kDa fragment, whereas cleavage by granzyme B leads to a fragment of 60 kDa. During necrosis, U1-70K is degraded to untraceable fragments, and oxidative fragmentation of U1-70K results in a smear of 33 – 38 kDa fragments. In conclusion, a cell can die in many ways, and specialized enzymes are activated in the different pathways. As a consequence, the creation of neo-epitopes directly depends on the type of cell death.
Susceptibility to modification may depend on the type of tissue

Most autoantigens, in particular those targeted in systemic autoimmune diseases, are evolutionarily conserved molecules with key functions in the cell, and can therefore be expected to be present in almost every cell. Consequently, one could imagine all autoantigens to be modified in dying cells and to be exposed to the immune system when a dying cell is not efficiently removed. But are all neo-epitopes present on these autoantigens? Ulanet et al. demonstrated that cleavage of the SSc autoantigen B23 (80) by granzyme B is cell type dependent (81). B23 was very efficiently cleaved in differentiated vascular smooth muscle cells upon incubation of the cells with granules containing granzyme B, whereas it was not cleaved in undifferentiated vascular smooth muscle cells or in fibroblasts that were treated in the same way. The SSc autoantigen DNA topoisomerase I, on the other hand, was susceptible to granzyme B cleavage in all of these cell types. These findings suggest that, although autoantigens might be released from dying cells, not all autoantigens will be modified in all cell types in the same way. Consequently, the generation of a neo-epitope on an autoantigen may not only depend on the cell death pathway that is entered, but also on the type of the dying cell.

Most experiments on cell death are performed in vitro using standard cell lines. In our studies initially HeLa cells and later Jurkat cells were used (Chapters 4, 6, 7 and 8). Jurkat cells were chosen for two reasons. First, their apoptotic cell death pathways have been well characterized, and second, Jurkat cells can grow in suspension, and thus can be cultured easily in relatively large quantities. Since standard cell lines are used instead of the cells that are actually affected in the patient, it should be kept in mind that the creation of neo-epitopes on autoantigens in vitro may differ from the in vivo situation. On the one hand, it would therefore be preferable to use the cells that are present in the inflamed tissue, as for example fibroblasts in connective tissue diseases. On the other hand, the tissue that is inflamed in the patient does not necessarily have to be the original source of the neo-epitopes. Given the fact that it is still unclear how and where a particular autoimmune disease is initiated, it will be very difficult, if not impossible, to choose the most relevant type of cell.

Genetic background

Normally, apoptotic cells are efficiently removed by, for example, macrophages, but accumulating evidence indicates that there is an impaired clearance of dying cells in some SLE patients. In these patients, apoptotic cells have been found to circulate in the blood (82) and in the germinal centres of lymph nodes (83), but apoptotic cells have also been described to accumulate in the skin after UV irradiation (84). An explanation for these findings could be that genetic defects are underlying the impaired removal of dying cells, certainly in cases when massive cell death (e.g. during an infection) occurs. Several mouse studies have pointed to genes that might play a role in this process. Indeed, mice deficient of factors involved in the removal of apoptotic cells, such as for example C57, DNase I, phosphatidylycerine receptor, C-reactive protein or secreted IgM, showed defects in clearance of apoptotic cells, developed SLE-like symptoms, and demonstrated production of SLE-associated antibodies (see reviews by (85-87)). Reduced levels or activities of such factors have also been observed in SLE patients (see reviews by (85-87)). Mutations in cell death-associated genes, such as Fas, Fas ligand and Bcl-2, have been associated with defective clearance as well, both in animal models (see review by (88)) and in patients (89,90).

Autoimmune diseases are genetically associated with several human leukocyte antigen (HLA) alleles (see Table 1). The HLA system is the general name of a group of genes in the major histocompatibility complex region on human chromosome 6 that are expressed on antigen-presenting cells, such as dendritic cells. They are involved in presenting antigens to T cells, which can subsequently activate B cells. Approximately 70% of RA patients produce detectable levels of autoantibodies to citrullinated antigens. Since HLA-DR4 is known to be associated with RA, it was postulated that HLA-DR4 could be involved in the presentation of citrulline-containing peptides. Indeed, Hill et al. demonstrated, using mice that were transgenic for human HLA-DR4, that a citrulline-containing peptide bound 100-fold stronger to the peptide-anchoring pocket of HLA-DR4, compared to the arginine variant of the same peptide (91). It is thought that, as a result, the modified (i.e. citrulline containing) peptides are presented in a higher density on the surface of antigen-presenting cells, which may exceed...
the biochemical margin for T cell activation (92).

In SSc, HLA genes are also correlated to the occurrence of specific autoantibodies and/or disease subsets. For example, anti-centromere antibodies have been associated with HLA-DQB1 alleles, and anti-DNA topoisomerase I autoantibodies with HLA-DRB1 alleles, the HLA-DRw1 allele and with a particular HLA-DQB1 sequence (see review by (93)). A recent study on immunogenetic risk and protective factors for polymyositis (PM) and dermatomyositis (DM) revealed that also in these diseases, HLA haplotypes are linked to autoantibody production (94). For example, the DRB1*0301 haplotype is correlated to the production of autoantibodies directed to the Jo-1 autoantigen. The DRB1*0701 and DQA1*0201, on the other hand, appeared to be protective for the development of anti-Jo-1 autoantibodies, but have been described to be a risk factor for the development of anti-Mi2 autoantibodies. It thus appears that autoantibody production in systemic autoimmune diseases is very much linked to particular HLA genes.

**Can recognition of specific fragments correlate with clinical symptoms?**

In 1997, Casciola-Rosen et al. postulated that susceptibility to cleavage by granzyme B was unique to SLE autoantigens (77). The authors demonstrated that several SLE autoantigens were cleaved by granzyme B, in contrast to non-SLE autoantigens, and that granzyme B cleavage resulted in fragments of different molecular weight compared to cleavage by caspases. In another study, the same authors hypothesized that oxidative fragmentation was specific for SSc autoantigens (77). They showed that SSc autoantigens are more readily fragmented by radicals than other autoantigens, and also oxidative fragmentation generated fragments of molecular weights that differed from cleavage by caspases or granzyme B. Simply taken, these findings suggested that granzyme B-initiated cell death processes would underlie autoantibody production in SLE, and that tissue damage by oxidative radicals would trigger autoantibody production in SSc. This hypothesis was supported by the idea that radical-mediated tissue injury specifically occurs after ischemia-reperfusion in the skin of patients suffering from SSc and Raynaud’s phenomenon (95,96). However, later studies demonstrated that granzyme B cleavage is not restricted to SLE autoantigens. For example, the SSc autoantigens centromere protein B, fibrillarin and DNA topoisomerase I are efficiently cleaved by granzyme B as well, whereas these proteins are much less susceptible to cleavage by caspases (53). Moreover, oxidative fragmentation appeared not to be restricted to SSc autoantigens, since also Ro60, an autoantigen in Sjögren’s syndrome and SLE, was shown to be antigenically altered upon oxidation (97). It was also shown that oxidatively modified nucleosomes exert enhanced binding to SLE autoantibodies (98). All these studies suggest that cell death processes create neo-epitopes that are involved in the production of autoantibodies.

**Several years ago, Greidingher et al. demonstrated in a very interesting series of experiments that sera from SLE patients with lupus skin disease (in these patients the skin becomes irritated and inflamed upon exposure to sun light) preferentially recognized the 40 kDa fragment of U1-70K that is generated upon cleavage by caspase-3. When cells are exposed to UV light in vitro, apoptosis is induced and U1-70K is cleaved by caspase-3 to this 40 kDa apoptotic fragment. Another subset of SLE patients, namely patients that also suffered from Raynaud’s phenomenon, preferentially recognized the 33-38 kDa smear of fragments derived from U1-70K upon treatment with radicals (78). These findings strongly suggested for the first time that the type of cell death, and thus the neo-epitopes that are created and exposed, not only is associated with specific autoantibody production but also with particular clinical symptoms.

In 2002, Schachna et al. (53) demonstrated that the majority of SSc patients suffering from ischemic digital loss (84%) preferentially recognized granzyme B-cleaved fragments of SSc autoantigens, as compared to their non-cleaved counterparts. In SSc patients without ischemic digital loss this preferential recognition occurred in only 40% of the patients. Five of 16 patients with ischemic digital loss demonstrated a stronger reactivity with a granzyme B-generated 60 kDa protein fragment, compared to reactivity with protein bands with higher molecular weights observed in the other patients. Although the identity of the 60 kDa fragment was not determined, it was suggested to be derived from centromere protein C (140 kDa). These findings also suggest that granzyme B cleavage generates novel epitopes on SSc autoantigens, and that the presence of autoantibodies directed to these epitopes correlates with clinical symptoms.

In conclusion, and as summarized in Figure 1, it is feasible that a combination of factors and events during (massive) cell death, including the enzymes that are activated, the type of tissue that is affected, the neo-epitopes that are formed and exposed, and the genetic background of the individual, determine whether an autoimmune reaction is triggered or not. To further study these mechanisms it will be important to identify additional cell death-associated neo-epitopes. Besides correlating the recognition of specific epitopes with clinical symptoms as was done in the study by Greidingher et al. (78), it would be of much value to correlate the recognition of specific epitopes with HLA alleles as well.

**How to gain more insight into the role of neo-epitopes?**

As a result of epitope spreading, autoimmune patient sera are in general highly polyclonal, i.e. directed to multiple epitopes, on one or more autoantigens (99,100). From the moment a patient enters the rheumatology clinic with first symptoms indicative of an autoimmune disorder, generally several months to years pass by before a final diagnosis is established. It is not difficult to imagine that, up to that moment, extensive epitope spreading may have taken place in these patients. It has been shown in several publications that autoantibodies can be detected in patient sera years before the actual onset of disease. In a large study, using the collection of the USA Department of Defense...
These findings illustrate that, when one wants blood in the years before diagnosis (102,103). With samples from patients that had donated the first clinical symptoms appear. This was be detected in RA patients many years before (up to 9.4 years earlier with a mean of 3.3 diagnosed with SLE. In 115 patients (88%) at samples of 130 individuals before they were Repository, Arbuckle et al. analyzed serum (103) attractive alternative approach to identify and approach would be to collect samples from patients with Raynaud's phenomenon, and to approach would be to start collecting serum samples, one approach to obtain such sera preferably before the first clinical symptoms. It is crucial to obtain sera as early as possible, preferably before the first clinical symptoms. Since it is difficult to obtain early serum samples, one approach to obtain such sera would be to start collecting serum samples from the moment the patient enters the clinic with the first symptoms, and to repeat this each time the patient visits the clinic. For example, approximately 9% of patients with primary Raynaud's phenomenon will eventually develop SSc (104), and sera from these patients could thus be regarded as early SSc sera. A workable approach would be to collect samples from patients with Raynaud's phenomenon, and to wait and see which patients develop SSc. It might be necessary to collect serum once or twice a year, as was performed in the study of anti-U1-70K reactivity described in Chapter 6. The disadvantage of such an approach is that collecting sufficient sera will take many years.

Helpful experimental systems to study the presence autoantibodies to cell death-specific epitopes are antibody phage display libraries from autoimmune patients, as demonstrated by the isolation of recombinant autoantibodies directed to apoptosis-specific epitopes on the U1-70K protein (Chapter 4). Another example is the isolation of autoantibodies to an early epitope on the SmB/B' protein from SLE libraries (105). The patient-derived antibody libraries used in the experiments described in this thesis were prepared from B cells harvested from peripheral blood and bone marrow tissue. As demonstrated in Chapter 6, the majority of anti-U1 snRNP positive patient sera contained autoantibodies to the apoptotic fragment of U1-70K, which is believed to be an early antigen in the anti-U1 snRNP response. These findings indicate that plasma cells secreting antibodies to early autoantigens might still be present in later stages of the disease, and such antibodies thus are present in the antibody repertoire of the libraries. Additionally, memory B cells are present in the bone marrow and as a consequence their repertoire is included in the libraries as well. The subtractive antibody phage display method described in Chapter 4 provides an attractive alternative approach to identify and study cell death-specific epitopes targeted in autoimmune diseases (see Figure 2c).

Figure 2 provides a schematic overview of possible approaches to identify novel neo-epitopes. Since it is rather difficult to obtain early sera, it is almost inevitable to use relatively late sera for most studies. To increase the chance of finding autoantibodies directed to cell death-specific epitopes among the majority of autoantibodies directed to "common" epitopes in a patient serum, competition experiments can be designed, in which autoantibodies directed to common epitopes are blocked with common epitopes. A straightforward method to study autoantibody reactivities is by immunoblotting using apoptotic (induced in various ways, including extrinsic and intrinsic pathways and granzyme B), necrotic, and oxidatively fragmented cell extracts (Figure 2a). Patient sera are preincubated with normal cell extract in solution, prior to the immunoblotting procedure, to compete for binding to common epitopes. The polypeptides that after the competition/selection procedure still are recognized by these sera are most likely detected via neo-epitope-specific autoantibodies. Alternatively, as depicted in Figure 2b, recombinant autoantigens can be expressed and treated with different cell death-specific enzymes, such as the enzymes listed in Table 2. After purification of the different cleavage fragments, analysis of autoantibody reactivities towards these fragments might directly provide insight into which enzymes are responsible for the creation of neo-epitopes on these autoantigens. Here, it would be advantageous to block autoantibody reactivity to common epitopes in a competition experiment as well.
How can cell death-specific epitopes be used for early diagnosis?

Current diagnostic laboratory tests are mainly based on the use of recombinant proteins or synthetic peptides as antigens. From the results described in Chapter 6, where it was demonstrated that apoptotic U1-70K is a superior marker compared to intact U1-70K because it is preferentially recognized by early sera, it is clear that detection of autoantibodies directed to cell death-specific epitopes could facilitate early diagnosis and consequently allow earlier treatment of patients. If a correlation between recognition of a specific neo-epitope and clinical symptoms can be established, (mild) treatment could be started to prevent or reduce clinical symptoms.

An emerging technology for autoantibody profiling is the autoantigen microarray (see review by (106)), which involves immobilization of a large number of different autoantigens (each in small amounts) on one chip. Microarray profiling enables the high throughput analysis of autoimmune patients. One of the advantages of this technique is that the reactivity of a patient serum can be profiled against a much broader range of autoantigens compared to conventional laboratory tests. In addition, recognition of sets of autoantigens can be correlated with subtypes of the disease (107,108). One could imagine that existing autoantibody microarrays can detect a broader range of antigens than conventional tests, and thus be a superior marker compared to intact U1-70K.

Figure 2. Possible approaches for the identification of novel cell death-specific epitopes. (a) Competition experiment on immunoblots. On the left, immunoblots of cell lysates treated with granzyme B, caspase-3 and radicals, and a control lysate, are incubated with patient serum and bound antibodies are detected using a labeled secondary antibody (not shown). If a specific protein fragment is stronger recognized than the intact form, this might indicate that autoantibodies in the serum are binding to a neo-epitope (depicted by a star in the figure) on this fragment. When the signal obtained from the fragment is equal or lower compared to the intact form, it is still possible that the fragment contains a neo-epitope and that specific autoantibodies react with it. Since the smaller fragment might lack some of the “common” epitopes, autoantibodies directed to these epitopes will not be detected on the fragment. When the patient serum is pre-incubated with the control lysate prior to the immunoblotting procedure (as depicted in (a) on the right), autoantibodies directed to common epitopes are bound in solution with these epitopes. Since the control lysate does not contain neo-epitopes, autoantibodies directed to neo-epitopes are not blocked in solution and will bind to the autoantigen fragment on immunoblot. In this example, U1-70K (70 kDa) is treated with granzyme B (resulting in a 60 kDa fragment), caspase-3 (40 kDa) and radicals (33-38 kDa smear), and all fragments are detected at the left. When the patient serum is pre-incubated with common epitopes present in the control lysates, the 70, 40, and 33-38 kDa fragments are no longer detected in contrast to the granzyme B-generated fragment of 60 kDa. This indicates that the particular patient has generated autoantibodies directed to a neo-epitope on U1-70K that is created upon treatment with granzyme B. (b) Competition experiment in ELISA. Autoantigens are recombinantly expressed and treated with different cell death-specific enzymes, such as granzyme B and caspase-3, or with radicals. Specific fragments containing the neo-epitopes (star) are purified and coated in the ELISA wells. Patient serum is pre-incubated with non-treated autoantigen to compete for binding to the common epitopes. After washing, bound antibodies (as depicted in the granzyme B well) can be detected with a suitable secondary antibody. (c) Isolation of specific recombinant autoantibody fragments by subtractive antibody phage display. Phages displaying recombinant antibody fragments from autoantibodies are incubated with non-biotinylated normal cell extract (1), prior to incubation with biotinylated lysates from dying cells (2). The biotin group is depicted as a yellow circle, labelled “b”. In this way, phages displaying antibody fragments directed to common epitopes are blocked for binding to the same epitopes on biotinylated proteins. After isolation and characterization of specific monoclonal antibodies, the protein that displays the specific epitope can be isolated by means of immunoaffinity purification, and can subsequently be identified by mass spectrometry.
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Color figures

a. mAbs: α P30′ + α Ker

Chapter 3, Figure 3 (page 47)

d. mAbs: α U1C + α RiboP + α Fibrin

Chapter 4, Figure 4 (page 61)

e. U1 snRNP (serum D18)

f. HeRNP C (serum B4)

g. PSF (mAb B91)

Chapter 7, Figure 4 (page 112)
Summary

Autoimmune diseases, which affect approximately 2-3% of the world population, are characterized by the occurrence of high titres of autoantibodies. Some of these autoantibodies are disease-specific and their presence is often a valuable diagnostic, and sometimes prognostic, tool for clinicians. For example, autoantibodies directed to citrullinated proteins are almost exclusively present in patients suffering from rheumatoid arthritis, and are therefore regarded as an excellent serological marker for this disease. The question why patients produce autoantibodies is still unsolved.

One model that is more and more supported by experimental data, hypothesizes that unusual posttranslational modifications, for example those occurring during cell death, can lead to the generation of neo-epitopes. These neo-epitopes are involved in breaking immunological tolerance to self-antigens. During the last 15 years the characterization of (auto)antibodies was greatly facilitated by the antibody phage display technology, a sophisticated methodology for the production of recombinant monoclonal antibodies. The goal of the studies described in this thesis was to design and develop novel applications of the antibody phage display technology (described in Part 1: Chapters 1 to 4) that can be used to gain more insight into the occurrence of autoantibodies and their antigenic targets.

Chapter 1 gives an overview of our current knowledge on the generation of antibodies, with special focus on the antibody phage display technology. In addition, it introduces the theory of the role of neo-epitopes in breaking immunological tolerance to self-antigens in patients suffering from an autoimmune disease.

Chapter 2 it is demonstrated that chickens can be immunized with a mixture of antigens to reduce the number of immune libraries and consequently to lower the number of experimental animals needed for the preparation of these libraries. Chickens were immunized with two protein mixtures, each containing seven distinct autoantigens. From the resulting immune libraries, antibodies directed to nine of these targets could be isolated. These recombinant antibodies can be applied in experiments where autoantibodies from patient sera are unsuitable, such as experiments involving antigen detection in human tissues, cells, or sera.

Chapter 3 recombinant antibodies were fused with human, mouse, and chicken immunoglobulin constant domains to allow easy double- and triple-staining of cells and tissues. This approach eliminates problems of co-staining with multiple monoclonal and polyclonal antibodies.

Chapter 4 describes a novel method to use antibody phage display for the discovery of disease markers. Phages from antibody phage libraries are first subtractively selected with biotinylated proteins from two different cell extracts, followed by a second selection on two-dimensional IEF/SDS-PAGE western blots. To demonstrate the feasibility of this method, phages from a mixture of antibody phage display libraries derived from patients with rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis (scleroderma), were selected based upon their reactivity with epitopes that are exclusively present in extracts made from apoptotic cells. Epitopes on three nuclear proteins, namely hnRNP C, p54\textsuperscript{nrb}, and U1-70K, could be identified as targets for the corresponding recombinant antibodies. They may thus represent autoantibodies associated with these autoimmune diseases. Furthermore, it could be shown that the recombinant anti-U1-70K antibody obtained from the patient libraries specifically interacted with an apoptosis-specific epitope on this autoantigen. Our findings strongly suggest that autoantibodies to apoptosis-specific epitopes on U1-70K are present in patient sera, which supports the hypothesis of neo-epitopes being involved in breaking tolerance to self proteins.

Autoantibodies directed to U1-70K, and other components of the U1 snRNP complex, are specifically associated with SLE and SLE overlap syndrome (mixed connective tissue disease). Chapter 5 gives an overview of the current knowledge on normal and cell death-specific posttranslational modifications that can be present on the U1-70K protein, and of the antibodies recognizing neo-epitopes generated by such modifications.

In Chapter 6 it is shown that the majority of anti-U1 snRNP positive patient sera (54%) preferentially recognize apoptotically cleaved U1-70K as compared to non-cleaved, intact U1-70K. Moreover, in a longitudinal study we showed that this preferential recognition is especially apparent early in the disease. These findings indicate (1) that autoantibodies to apoptosis-specific epitopes on U1-70K are actually present in patient sera, (2) that these antibodies appear before autoantibodies directed to the intact form are present, and (3) that the apoptosis-specific epitopes are involved in breaking immunological tolerance to U1-70K and the U1 snRNP complex.

Besides U1-70K, two other potential cell death-specific targets for autoantibodies were identified in Chapter 4, namely hnRNP C and p54\textsuperscript{nrb}. In the experiments described in Chapter 7 the apoptotic modifications on hnRNP C and p54\textsuperscript{nrb} were further studied by immunoblotting and immunofluorescence assays, using normal and apoptotic cells. The results demonstrated that p54\textsuperscript{nrb} is not only cleaved, but also undergoes a shift in isoelectric point during apoptosis, findings that were not described before. In the case of hnRNP C1, five isoforms could be detected in normal cell extracts, whereas in apoptotic cells only three isoforms were detectable. In addition, hnRNP C and PSF were shown to translocate from the nucleoplasm in healthy cells to apoptotic
bodies on the cell surface in apoptotic cells. Translocation of these modified proteins to the cell surface may enhance their exposure to the immune system and facilitate autoantibody formation. HnRNP C had been described previously as an autoantigen in a few patients. In this chapter it is demonstrated that p54nrb is an autoantigen as well, because autoantibodies directed to p54nrb were found in patients suffering from polymyositis/scleroderma overlap syndrome.

Oxidative stress and the formation of free radicals are believed to play a role in the pathology of scleroderma. In Chapter 8 the autoantibody reactivity of sera, obtained from patients with scleroderma or scleroderma-associated disease, with neo-epitopes that are created by radicals is addressed. Autoantibody reactivity was analyzed by immunoblotting using either normal cell extracts or cell extracts that were treated with Fe2+ and ascorbic acid. Twelve patient sera were identified that differentially reacted with proteins in the two types of extracts. It is hypothesized that the oxidatively modified proteins display neo-epitopes that play a role in breaking tolerance to these self-proteins in these patients. Two sera were further analyzed and it was demonstrated that the neo-epitopes recognized by these sera in oxidatively modified cell extracts are not created during well-known forms of cell death, such as apoptosis or necrosis.

The results of the experiments described in this thesis and some remaining questions are discussed in Chapter 9.

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**Summary**

Auto-immunziekten, waar 2-3% van de wereldbevolking aan lijdt, worden gekenmerkt door de aanwezigheid van hoge titers aan auto-antilichamen. Sommige van deze auto-antilichamen zijn specifiek voor één bepaalde auto-immuunziekte en hun aanwezigheid is daardoor vaak een waardevol diagnostisch, en soms voorspellend, hulpmiddel voor artsen. Auto-antilichamen gericht tegen gecitrullineerde eiwitten komen bijvoorbeeld vrijwel alleen voor in patiënten met reumatoïde artritis en worden daarom beschouwd als uitstekende serologische merkers voor deze ziekte. De vraag waarom patiënten auto-antilichamen maken, blijft vooralsnog onbeantwoord. Een model dat door steeds meer experimentele gegevens wordt ondersteund, veronderstelt dat ongebruikelijke post-translationele veranderingen van lichaamseigen eiwitten, bijvoorbeeld veranderingen die plaatsvinden tijdens celdood, kunnen leiden tot de vorming van zogenaamde neo-epitopen. Zulke neo-epitopen kunnen vervolgens leiden tot het doorbreken van immunologische tolerantie voor lichaamseigen antigenen in patiënten die lijden aan een auto-immuunziekte.

Antilichaam-faagdisplaybanken kunnen gemaakt worden van niet-geïmmuniseerde en geïmmuniseerde individuen of proefdieren. Over het algemeen hebben antilichamen die geïsoleerd worden uit banken van geïmmuniseerde proefdieren een hogere affiniteit dan antilichamen uit naïeve of synthetische (d.i. niet-geïmmuniseerde) banken. Daarnaast is het maken van immuunbanken van kippen minder werk dan het maken van zulke banken van humaan of muizenmateriaal. In Hoofdstuk 2 laten we zien dat kippen ook geïmmuniseerd kunnen worden met een mengsel van verschillende antigenen met als doel het aantal te synthetiseren immuunbanken en daarmee ook het aantal kippen dat hiervoor gebruikt moet worden, te beperken. Kippen werden geïmmuniseerd met twee eiwitmengsels die elk zeven verschillende eiwitantigenen bevatten. Uit de resulterende immuunbanken konden antilichamen tegen negen van deze antigenen worden geïsoleerd. De geselecteerde

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**Samenvatting**

Auto-immuunziekten, waar 2-3% van de wereldbevolking aan lijdt, worden gekenmerkt door de aanwezigheid van hoge titers aan auto-antilichamen. Sommige van deze auto-antilichamen zijn specifiek voor één bepaalde auto-immuunziekte en hun aanwezigheid is daardoor vaak een waardevol diagnostisch, en soms voorspellend, hulpmiddel voor artsen. Auto-antilichamen gericht tegen gecitrullineerde eiwitten komen bijvoorbeeld vrijwel alleen voor in patiënten met reumatoïde artritis en worden daarom beschouwd als uitstekende serologische merkers voor deze ziekte. De vraag waarom patiënten auto-antilichamen maken, blijft vooralsnog onbeantwoord. Een model dat door steeds meer experimentele gegevens wordt ondersteund, veronderstelt dat ongebruikelijke post-translationele veranderingen van lichaamseigen eiwitten, bijvoorbeeld veranderingen die plaatsvinden tijdens celdood, kunnen leiden tot de vorming van zogenaamde neo-epitopen. Zulke neo-epitopen kunnen vervolgens leiden tot het doorbreken van immunologische tolerantie voor lichaamseigen antigenen. De afgelopen vijftien jaar heeft de antilichaam-faagdisplaytechnologie, een geavanceerde methode voor het maken van recombinante monoklonale antilichamen, het karakteriseren van (auto-)antilichamen makkelijker gemaakt. Het doel van de studies die beschreven zijn in dit proefschrift was om nieuwe toepassingen van de antilichaam-faagdisplaytechnie te ontwerten en te ontwikkelen (beschreven in deel 1: Hoofdstukken 1 tot en met 4) om meer inzicht te krijgen in de aanwezigheid van auto-antilichamen en hun corresponderende antigenen (beschreven in deel 2: Hoofdstukken 5 tot en met 8).

**Hoofdstuk 1** geeft een overzicht van onze huidige kennis op het gebied van het maken van antilichamen, waarbij de nadruk ligt op de antilichaam-faagdisplaytechnologie. Daarnaast geeft dit hoofdstuk een inleiding in de theorie over de rol die neo-epitopen kunnen spelen in het doorbreken van immunologische tolerantie voor lichaamseigen antigenen in patiënten die lijden aan een auto-immuunziekte.

Antilichaam-faagdisplaybanken kunnen gemaakt worden van niet-geïmmuniseerde en geïmmuniseerde individuen of proefdieren. Over het algemeen hebben antilichamen die geïsoleerd worden uit banken van geïmmuniseerde proefdieren een hogere affiniteit dan antilichamen uit naïeve of synthetische (d.i. niet-geïmmuniseerde) banken. Daarnaast is het maken van immuunbanken van kippen minder werk dan het maken van zulke banken van humaan of muizenmateriaal. In **Hoofdstuk 2** laten we zien dat kippen ook geïmmuniseerd kunnen worden met een mengsel van verschillende antigenen met als doel het aantal te synthetiseren immuunbanken, en daarmee ook het aantal kippen dat hiervoor gebruikt moet worden, te beperken. Kippen werden geïmmuniseerd met twee eiwitmengsels die elk zeven verschillende eiwitantigenen bevatten. Uit de resulterende immuunbanken konden antilichamen tegen negen van deze antigenen worden geïsoleerd. De geselecteerde
recombinante antilichamen kunnen worden toegepast in experimenten waarvoor auto-
antilichamen uit patiëntensera niet geschikt zijn, zoals experimenten waarbij auto-
antigenen in humane weefsel, cellen of sera geanalyseerd moeten worden.

Recombinante antilichamen uit anti-
liachaam-faagdisplaybanken zijn toepasbaar in een breed scala aan assays en zijn daarom waardevolle onderzoekshulpmiddelen. In Hoofdstuk 3 werden recombinante anti-
liachaam gefuseerd met constante domeinen van humane, muizen- en kippen-antilichamen om twee- en drievoudige kleuringen van cellen en weefsel te vergemakkelijken. Deze aanpak omzeilt de problemen die er kunnen zijn bij het gelijktijdig kleuren van weefsel met meerder mono- en polyklonale antilichamen.

Hoofdstuk 4 beschrijft een nieuwe methode om met behulp van antilichaam-faag-
display ziektemarkers te ontdekken. Fagen worden uit antilichaam-faagdisplaybanken geïsoleerd door eerst substraat te selecteren op gebitinylleerde eiwitten in twee verschillende extracten en vervolgens een tweede selectie te doen op tweedimensionale IEF/SDS-
PAGE western blots. Om te laten zien dat deze methode werkt, worden fagen uit een mengsel van banken van patiënten met reumatoïde artritis, systemische lupus erythematodes en systemische s❝erose (sclerodermie) geselecteerd op reactiviteit met epitopen die een rol spelen bij het doorbreken van immunologische tolerantie voor lichaamseigen eiwitten. Auto-antilichamen tegen U1-70K en tegen andere componenten van het U1 snRNP-
complex komen voornamelijk voor bij SLE en het SLE-overlap-syndroom. Hoofdstuk 5 geeft een overzicht van de huidige kennis op het gebied van normale en eeldood-specifiede post-translationele veranderingen die aanwezig kunnen zijn op het U1-70K eiwit en van de auto-antilichamen die zulke potentiële neo-epitopen herkennen. In Hoofdstuk 6 wordt aangetoond dat de meerderheid (54%) van sera met auto-
antilichamen gericht tegen het U1 snRNP-complex, bij voorkeur het apoptotisch gekliefde U1-70K herkennen ten opzichte van het niet-
gelijktijdig, duidelijk U1-70K eiwit. Bovendien laten we in een longitudinale studie zien dat deze preferentiële herkenning voornamelijk in een vroeg stadium van de ziekte optreedt. Deze bevindingen duiden er sterk op dat (1) auto-antilichamen tegen apoptose-
specifieke epitopen op U1-70K daadwerkelijk voorkomen in sera van patiënten, (2) dat deze auto-antilichamen tegen apoptose-
specifieke epitopen verschijnen vóórdat auto-antilichamen tegen de intacte vorm van U1-70K aanwezig zijn en (3) dat de apoptose-
specifieke epitopen betrokken zijn bij het doorbreken van immunologische tolerantie voor U1-70K en het U1 snRNP complex. Naast U1-70K werden twee andere mogelijke merkers voor auto-immunziekten geïdentificeerd in Hoofdstuk 4, namelijk de kerneiwitten hnRNP C en p54
/PSF. In de experimenten die beschreven zijn in Hoofdstuk 7 werden de apoptotische veranderingen op deze eiwitten bestudeerd met behulp van immunoblotting- en immunofl uorescentietechnieken. Hiervoor werden zowel gezonde als apoptotische cellen gebruikt. Er werd aangetoond dat p54
/PSF niet alleen gekliefd wordt tijdens apoptose, maar dat tevens het isoëlectrische punt verandert. Deze bevinding was nog niet eerder beschreven. In het geval van hnRNP C1 bleken vijf isovormen aanwezig te zijn in het celextract van gezonde cellen, terwijl er maar drie isovormen detecteerbaar waren in celextracten van apoptotische cellen. Daarnaast werd aangetoond dat hnRNP C en PSF zich tijdens apoptose van hun normale localisatie, het nucleoplasma, verplaatsen naar apoptotische blaasjes aan het oppervlak van de cel. De verplaatsing van deze veranderde eiwitten naar het celoppervlak zou hun blootstelling aan het immuunssysteem kunnen versterken en daardoor de productie van auto-
antilichamen vergemakkelijken. HnRNP C was al eerder beschreven als een auto-antigeen in een aantal patiënten. Daarnaast konden we in dit hoofdstuk aantonen dat ook p54
/PSF een auto-antigeen is, omdat auto-antilichamen tegen p54
/PSF blijven voor te komen in patiënten die lijden aan het polymyositis/sclerodermie-
overlap-syndroom.

Er wordt verondersteld dat oxidatieve stress en de productie van vrije radicalen een rol spelen bij de pathologie van sclerodermie. In Hoofdstuk 8 wordt in sera van patiënten met sclerodermie of sclerodermie-gereleatete ziekten de reactiviteit bekeken van auto-
antilichamen met neo-epitopen die ontstaan door radicalen. Serumreactiviteit werd geanalyseerd op western blots, waarbij gebruik werd gemaakt van controle-extracten en van celextracten die behandeld waren met Fe
/Fe
 en ascorbinezuur. Twaalf patiëntensera konden worden geïdentificeerd die verschillend reageerden met eiwitten in de twee extracten. Het lijkt er dus op dat ook oxidatief-veranderde eiwitten neo-epitopen kunnen bevatten die een rol spelen bij het doorbreken van tolerantie voor deze lichaamseigen eiwitten in deze patiënten. Twee sera werden meer in detail geanalyseerd en er werd aangetoond dat de neo-epitopen die door deze sera worden herkend in het celextract dat behandeld was met Fe
 en ascorbinezuur niet gecreëerd worden tijdens de bekende vormen van celdood, zoals apoptose en necrose.

De resultaten van de experimenten die in dit proefschrift zijn beschreven worden samen met een aantal nog onbeantwoorde vragen bediscussieerd in Hoofdstuk 9.
Het proefschrift is af! Ook al staat alleen mijn naam op de omslag van dit boekje, het was niet tot stand gekomen zonder de hulp van vele anderen. Gelukkig is er het veel-gelezen Dankwoord om daar even bij stil te staan.

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Daniëlle

Van augustus 2001 tot augustus 2002 was zij werkzaam als analiste op de afdeling Biochemie van de Radboud Universiteit Nijmegen. Vervolgens voerde zij van augustus 2002 tot en met april 2006 haar promotieonderzoek uit als junior onderzoeker op dezelfde afdeling, onder begeleiding van Dr. Jos M.H. Raats, Prof. Dr. Ger J.M. Pruijn en Prof. Dr. Walther J. van Venrooij. Uit het onderzoek dat zij in deze periode uitvoerde, vloeide dit proefschrift voort.

Sinds 1 mei 2006 is zij werkzaam als postdoc onderzoeker bij het Instituut voor Klinische Chemie aan de Universiteit van Zürich, onder begeleiding van Prof. Dr. Arnold von Eckardstein.

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