Review

Death, autoantigen modifications, and tolerance

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Abstract

Autoantibodies present in the serum of patients with a variety of inflammatory diseases have proven useful as diagnostic markers and as probes with which to elucidate biochemical and signaling pathways. The mechanisms governing the generation of autoantibodies remain elusive, constituting a critical missing link in our understanding of rheumatologic illnesses. Several lines of experimentation in recent years have strongly implicated events surrounding cell death in this process. This review will address the potential role played by death-specific modifications of autoantigens in bypassing tolerance to highly conserved autoantigens, including nucleic acids, lipids, and proteins.

Keywords: apoptosis, autoantibody, autoimmunity, modification, tolerance

Introduction

A hallmark of autoimmune diseases such as systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis, type I (insulin-dependent) diabetes mellitus, and dermatomyositis is the production of highly specific autoantibodies that recognize evolutionarily conserved molecules. The mechanisms by which these largely intracellular molecules are recognized as foreign are poorly understood. Many recent studies have implicated an important role for cell death processes in mediating the bypass of tolerance to these autoantigens. Since this subject was last reviewed, a number of new autoantigen modifications that accompany apoptotic and nonapoptotic cell death have been described [1,2]. This review serves as an update on this expanding field, and defines new areas of research that should be explored in order to resolve this important scientific conundrum.

Brief history of apoptosis and autoantibodies

Apoptosis and its role in the development of autoimmunity have been extensively reviewed by several authors [1–8], and the reader is referred to that work for a more comprehensive review of the topic. Apoptosis is a morphologically and biochemically defined form of cell death that plays a significant role in the deletion of autoreactive lymphocytes, removal of cells infected with virus, elimination of cancerous cells, and embryogenesis of complex multicellular organisms. As one might expect, defects in cell death have been implicated in the development of autoimmune diseases, persistent viral infection, malignancy, and developmental defects.

That apoptosis might play an important role in bypassing tolerance to intracellular autoantigens was first demonstrated by LeFeber *et al* in 1984 [9••]. Those investigators

APC = antigen-presenting cell; CTL = cytotoxic T lymphocyte; ICP = infected cell protein; MCTD = mixed connective tissue disease; MHC = major histocompatibility complex; ORF = open reading frame; PARP = poly A ribose polymerase; SLE = systemic lupus erythematosus; snoRNP = small nuclear ribonucleolar protein; SRPK = serine arginine protein kinase; UL6 = unique region protein 6.

observed that nuclear antigens that are present in cultured human keratinocytes derived from neonatal foreskin relocalized after exposure to ultraviolet irradiation. Several antigens including Ro, small nuclear ribonuclear protein (snRNP), and Smith complex relocalized from their normal nuclear address to the cell surface membrane. This work was confirmed and extended by Golan *et al* in 1992 [10•] when they demonstrated that keratinocytes derived from the skin of SLE patients avidly bound autoantibodies at their cell surface membrane following ultraviolet A and ultraviolet B exposure. This occurred in a less dramatic manner when the keratinocytes were derived from healthy control patients. These experiments suggested that keratinocytes from SLE patients were significantly more sensitive to ultraviolet light, which is an important cause of SLE dermatologic manifestations. This correlated with the observed relocalization of autoantigens to a locale where they might be readily accessible to components of the immune system, including lymphocytes and antigen-presenting cells (APCs).

The morphologic features of apoptotic cell death had been described over a decade before these important reports [11••]. However, it was not until the now seminal experiments performed by Casciola-Rosen *et al* [12••] were completed that an important discovery was made – that ultraviolet-irradiated keratinocytes were in fact undergoing apoptosis. The autoantigens were shown to cluster in two discrete cell surface 'membrane blebs'. The larger blebs (called apoptotic bodies) contained predominantly Ro, La, snRNPs, and nucleosomal DNA. The smaller structures were recognized by autoantibodies specific for endoplasmic reticulum components, as well as Ro and ribosomal components [12••]. The same group of investigators also showed that the cell is further modified by the increased external cell surface expression of phosphatidylserine, a procoagulant that has been implicated in the antiphospholipid antibody syndrome [13].

Interestingly, several other apoptotic stimuli lead to autoantigen relocalization, including infection of cells with Sindbis virus [14]. Sindbis viral particles colocalize with ribosomal and endoplasmic reticulum components exclusively in small blebs, generating packages of autoantigens that are closely associated with viral proteins. Other molecules have been observed in association with keratinocyte surface blebs, including complement C1q (complete deficiency of which is almost uniformly associated with SLE) [15]. The clustering of autoantibodies on the surface of apoptotic cells has also been described for antineutrophil cytoplasmic autoantibodies, a specific marker for Wegener's granulomatosus. Granules of apoptotic, but not untreated neutrophils bind antineutrophil cytoplasmic autoantibodies in a region immediately beneath the intact cell membrane [16]. These studies demonstrate a second important piece to the autoantibody puzzle – not only are the autoantigens in locations where they ordinarily are not present, but they are differentially packaged in a manner that may partly explain the diversity and combination of autoantibody profiles that characterize SLE and subsets of SLE.

In addition to their intracellular relocalization in response to stressful stimuli, many autoantigens are specifically modified by enzymes that are activated as part of the cell death program. For example, at least 38 autoantigens are substrates for nearly a dozen mammalian and viral proteases (Table 1). Some antigens are nonproteolytically modified (eg by kinases and phosphatases), whereas other autoantigens are directly modified by toxins such as mercury, presumably by processes that are enzyme-independent (Table 2). This extensive list of autoantigen modifications, and the specific roles that they may play in generating molecules that are recognized as foreign by the immune system, are the focus of the remainder of the present review.

Modifications of autoantigens in association with apoptotic cell death

Modifications of chromatin components

A biochemical hallmark of apoptotic cell death is the cleavage of DNA into oligosome-sized fragments, called 'DNA ladders' when analyzed by ultraviolet illumination of ethidium-stained agarose gels. The molecular details of this process have now been elucidated and are reviewed in detail elsewhere [17•]. Chromatin modified in this way is present in apoptotic blebs, together with protein autoantigens [12••]. Anti-DNA antibodies are intimately associated with SLE, and their presence has both diagnostic and prognostic significance (reviewed in [18•]). Before the general acceptance of DNA 'laddering' as an important characteristic of apoptosis, it was observed that serum derived from human SLE patients contained DNA that had been similarly processed, whereas 'DNA ladders' were absent from healthy control sera [19]. Analysis of serum from young MRL/*lpr*/*lpr* mice [20] has yielded similar results.

If sera from SLE patients contains circulating apoptotic debris, from where does it arise? Cells derived from SLE patients have been reported to undergo apoptosis spontaneously at a faster rate, and some apoptotic cells, including peripheral blood neutrophils and lymphocytes, circulate at higher levels in the blood of SLE patients [21,22]. This phenomenon appears to be a unique characteristic of cells from SLE patients, because most other diseases associated with an excess of apoptotic cells (eg acquired immune deficiency syndrome and systemic vasculitis) are generally not associated with high titers of specific autoantibodies [23]. Circulating nucleosomes can be detected in the blood of patients undergoing radiation therapy or chemotherapy, however [24]. Although unique autoantibodies have been described in association with

specific malignancies, none are known to recognize proteins that are modified during cell death [25–28].

In addition to cleavage of internucleosomal DNA, the question also arises regarding whether chromatin and associated proteins might be modified in other ways. A recent report [29] suggested that 'apoptotic nucleosomes' isolated from cell supernatants of apoptotic hybridoma cells contain degraded histone H3 and H4. Another report [30] suggested that DNA methylation and deoxycytosine/deoxyguanine content of nucleosomes prepared from apoptotic lymphocytes is also abnormal. To date no other specific modifications of nucleosomes have been reported to occur as a result of apoptosis. Taken together, these studies suggest that chromatin that is modified during apoptosis may circulate, either in native form or packaged in apoptotic bodies, in the serum of patients with a variety of systemic autoimmune diseases. Clearly other factors are required for the apoptotic material to serve as an immunogen in SLE patients and autoimmune mice.

Nucleolytic degradation of RNA

To date, four RNA molecules have been identified as substrates for ribonuclease(s) that are activated during apoptosis (for review [17•]). These include the 18S and 28S ribosomal RNAs, the Ro-associated Y RNAs, and the U1-snRNA molecule. Each of these RNA molecules is associated with particles that are common targets of the immune response in SLE and mixed connective tissue disease (MCTD) [18•]. The details of these discoveries have been extensively covered in an excellent recent review [17•] and will be touched on only briefly here.

The 18S ribosomal RNA was shown to undergo unique cleavage in response to DNA-damaging stimuli such as γ-irradiation [31,32]. Other than the 72-kDa subunit of the signal recognition particle and the L10E ribosomal protein, both of which are cleaved during apoptosis, the 18S and 28S ribosomal RNA molecules are the only other constituents of the ribosome that are known to undergo an apoptosis-specific modification [33,34]. The Y RNA molecules, small RNAs that exist in complex with the Ro autoantigen in the cytoplasm, are degraded in response to a number of stressful stimuli [35]. Cleavage is caspase-dependent and is inhibited by zinc, small peptide caspase inhibitors, and bcl-2. Ro remains bound to the degradation products, protecting a highly conserved region of the Y RNA. The final RNA moiety that is known to be cleaved, the U1-snRNA molecule, also remains associated with protein constituents of the U1-snRNP [36]. These constituents, which include Smith complex proteins found in several snRNPs, as well as U1-snRNP-specific proteins such as U1-70 kD and U1A, are major targets of the immune response in SLE and MCTD, respectively. What role cleavage of these RNA moieties plays in the development of an immune response to components of these particles is currently unknown, but antibodies capable of directly recognizing individual RNAs have been described (for review [17•]). The nuclease(s) responsible for these cleavage events presently remain unidentified, as are the effects that RNA cleavage may have on cell death pathways.

Caspase-mediated protein cleavage

The major 'executioners' of cell death are 'caspases', a family of cysteine proteases that cleave substrate proteins immediately after aspartic acid residues (for review [37•]). At least 14 members of this protease family have been described. Some of the earliest caspase substrates to be identified were already known to be autoantigens, suggesting to these pioneering investigators that cleavage of autoantigens during cell death might contribute to their immunogenicity [12••,38]. Four major screening assays have been reported that attempted to identify autoantigens that are cleaved during apoptosis. Three of these used human autoimmune sera as probes to identify cleaved proteins by Western blotting [12••,39,40]. The fourth study [41] employed sera to immunoprecipitate autoantigen particles from lysates prepared from radiolabeled apoptotic cells. A number of other autoantigens have been identified as caspase cleavage substrates by other investigators studying other systems. The results of these screens have been summarized in several recent reviews and are compiled in Table 1 [1].

Autoantigen phosphorylation

Many proteins are recognized by autoantibodies but are not substrates for apoptotic proteases such as caspases and granzymes (see Autoantigen cleavage by cytotoxic T lymphocyte granule proteases below). In an attempt to identify other post-translational modifications of autoantigens that might render them immunogenic, we screened a large number of human and mouse autoimmune sera for the ability to precipitate novel phosphoproteins from radiolabeled apoptotic Jurkat cell lysates [41,42]. Almost all lupus sera are capable of precipitating new phosphoproteins in such an assay, suggesting that this autoantigen modification might also be of importance [41]. Of the eight phosphoproteins initially discovered in this way, we have definitively identified four of the proteins as members of the serine arginine family of RNA splicing factors [41,42]. Serine arginine proteins are critical regulators of constitutive and alternative messenger RNA splicing (for review [43•]). Their splicing activity is regulated by reversible serine phosphorylation of their carboxyl-terminal serine arginine motifs by a number of interesting kinases, including the serine arginine protein kinases SRPK1 and SRPK2, and the scleroderma autoantigen topoisomerase I [44–48]. The SRPK activity of topoisomerase I has only recently been discovered and characterized [48]. Intriguingly, SRPK1 and topoisomerase I are cleaved by caspases during apoptosis, suggesting that their proteolysis

Table 1

Proteolytic cleavage of autoantigens during apoptosis

Autoantigen	Function	Cleavage site	Protease	Fragment size	Disease	References
Actin	Cytoskeleton	LVID^{11}, ELPD^{244}	1	41,30,14	Autoimmune Hepatitis	[95]
Alanyl tRNA synthetase	Translation	(VAPD^{632})	GB	58	PM/DM, ILD	[18•,51••]
CENP-B	Centromere protein	(VDSD^{457})	GB	58,40	Scleroderma	[18•,51••]
DNA-PK	DNA repair	DEVD^{2712} VGPD^{2698}	3 GB	250,165 250,150	SLE, Scleroderma, Overlap Syndrome	[37•,96] [69••]
Fibrillarin	snoRNP protein	VGPD^{184}	GB	37	SLE, Scleroderma, Overlap Syndrome	[51••]
α-Fodrin	Actin binding protein	?	Caspases Calpain	150,120	Sjögren's Syndrome	[97]
Histidyl tRNA synthetase	Translation	(LGPD^{48})	GB	40	PM, ILD, DM, Overlap Syndrome	[18,51••]
Histone H3	DNA core protein	RKQL^{20}A	FMDV 3C^{pro}	13	SLE	[86]
hnRNP A1	RNA processing	?	?	32,29,16	SLE, RA, MCTD	[34,98]
hnRNP C1	RNA processing	?	3,6,7	40	Scleroderma, Psoriasis	[99]
hnRNP C2	RNA processing	?	3,6,7	40	Scleroderma, Psoriasis	[99]
hsp-90	Stress response	(DEED^{259})	?	54	SLE	[18•,100]
Isoleucyl tRNA synthetase	Translation	(VTPD^{983})	GB	?	PM/DM, ILD	[18•,51••]
Keratin	Cytoskeleton	VEVD^{238}	3,6,7 Calpain	26,22,19	GVHD, DLE	[18•,100,101]
Ki-67	Cell proliferation	(VCTD^{1481})	GB	167,148	SLE	[18•,51••]
Ku-70	DNA replication, repair	(ISSD^{79})	GB	?	SLE, PM/Scleroderma	[18•,51••]
La	Pol III transcription	(DEHD^{371}),(DEHD^{374}) LEED^{220} VQFQ^{358}G	Not 1,2,3,8,9 GB PV 3C^{pro}	45 ? 50	SLE	[54] [51••] [84]
Lamin A	Nuclear skeleton	VEID^{230}	6	45	SLE-like Disease, APLA	[102,103]
Lamin B	Nuclear skeleton	(EEID^{448}) (VEVD^{231})	?	45,28	SLE-like Disease, APLA	[40,104]
Lamin C	Nuclear skeleton	VEID^{230}	6	45	SLE-like Disease, APLA	[102,103]
Mi-2	DNA methylation, chromatin remodeling	VDPD^{1312}	GB	75,72,48	DM	[51••]
Nucleolin	Nucleolar RNA binding protein	? ?	? GA	16 88	SLE	[34,105] [70]

Table 1

Continued

Autoantigen	Function	Cleavage site	Protease	Fragment size	Disease	References
NuMA	Mitosis	? VATD^{1705}	3,4,6,7,8 GB	180,160 175	Sjögren's Syndrome	[40,106] [51••]
PARP	DNA binding protein	DEVD^{216} VDPD^{536}	1,2,3,6 GB	85,31 M (89,72,62)	SLE	[40,107,108] [69•]
PM-Scl	Exoribonuclease	(VEQD^{252})	GB	85,74	PM, Scleroderma	[18•,51••]
PMS1	DNA repair	ISAD^{496}	GB	50,60	?	[51••]
PMS2	DNA repair	VEKD^{493}	GB	60,50,36	?	[51••]
RNA helicase A	RNA processing, transcription	(DTPD^{96})	3	M (120-130)	SLE	[109]
RNA polymerase I	RNA synthesis	(ICPD^{448})	GB	?	Scleroderma	[18•,51••]
RNA polymerase II	RNA synthesis	(ITPD^{370})	GB	190,110,92	Scleroderma	[18•,51••]
SP1	Transcription	NSPD^{584}	3,7	68,45,22	UCTD	[110]
SRP-72	Protein translation, ER localization	(SELD^{617}) (VTPD^{573})	3 GB	66 62	DM/PM	[33] [51••]
Topoisomerase I	DNA unwinding, SR protein kinase	DDVD^{146} EEED^{170} PEDD^{123} IEAD^{15}	3 3 6 GB	M (76-82) 98,75,72	Scleroderma, PM	[40,49] [51••]
Topoisomerase II	DNA unwinding	?	?	M (125-160)	SLE, Fibrosing Alveolitis	[40]
Transglutaminase	Protein cross-linking	?	3	48	Coeliac Disease	[59,60]
UBF/NOR-90	Nucleolar transcription factor	? (VRPD^{220})	? GB	M (24,32,35,55) ?	Sjögren's Syndrome, Scleroderma	[40] [51••]
U1-70 kD	RNA splicing	DGPD^{341} LGND^{409}	3 GB	40, 22 60	SLE, Scleroderma, MCTD	[38,96]
Vimentin	Cytoskeleton	IDVD^{259} DSVD^{85} ?	1,2,3,8,12 HIV-1 protease	44,36,25,15 ?	SLE, BD, RA, Sjögren's Syndrome	[104,111–113] [85]

The 'Cleavage site' column lists sites that have been definitively identified, either by mutational analysis of the substrate P1 aspartic acid residue, or by peptide sequencing of proteolytic fragments. Sequences in parentheses signify untested but likely cleavage sites. The fourth column lists proteases that have been implicated in the cleavage reaction. Caspases are listed by number. The substrate is susceptible to direct cleavage by the indicated recombinant caspase or by purified calpain or GB in an *in vitro* cleavage assay. The fifth column signifies molecular weights (in kDa) of cleavage products as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The acronyms used in this table (and not included in the abbreviations list at the start of the review) are listed in the appendix.

Table 2

Other death-associated autoantigen modifications

Autoantigen	Function	Modification	Disease	Reference
Actin	Cytoskeleton	Transglutamination	Autoimmune Hepatitis	[58,95,114]
DNA/nucleosome	Genetic code	DNA cleavage DNA methylation	SLE	[18•,19,30]
Fibrillarin	snoRNP component	Oxidative fragmentation	Scleroderma	[71•]
Histone H2A	DNA core	Ubiquitin conjugation/deconjugation	SLE	[19,62,63]
Histone H2B	DNA core	Transglutamination	SLE	[19,58]
La	Pol III transcription	Dephosphorylation	SLE	[54]
PARP	DNA binding protein, DNA repair	ADP-ribosylation Necrotic fragmentation	SLE	[57] [76]
pp90	?U2-snRNP protein	Phosphorylation	SLE	[42]
pp34	SR splicing factor	Phosphorylation	SLE	[41,42]
pp46	?	Phosphorylation	SLE	[41]
pp17	?	Phosphorylation	SLE	[41]
Ribosomal RNA	Translational apparatus	RNA cleavage	SLE	[31,32,115]
SRp40	SR splicing factor	Phosphorylation	SLE	[42]
SRp55	SR splicing factor	Phosphorylation	SLE	[42]
SRp20	SR splicing factor	Phosphorylation	SLE	[42]
Topoisomerase I	DNA unwinding, SR protein kinase	Necrotic fragmentation Oxidative fragmentation	Scleroderma	[76] [71•]
Topoisomerase II	DNA unwinding	Ubiquitin conjugation/deconjugation	SLE, Fibrosing Alveolitis	[40,64,116]
Troponin	Muscle function	Transglutamination	Necrobiosis Lipoidica	[58,117]
Tubulin	Cytoskeleton	Transglutamination	SLE	[58,118]
U1-70 kD	mRNA splicing	Necrotic fragmentation	SLE, MCTD	[76]
U1-snRNA	mRNA splicing	RNA cleavage	SLE, MCTD	[36]
UBF/NOR-90	Nucleolar transcription factor	Oxidative fragmentation Necrotic fragmentation	Sjögren's Syndrome, Scleroderma	[71•] [76]
Vimentin	Cytoskeleton	Citrullination	SLE, BD, RA, Sjögren's Syndrome	[67]
Y RNA	Unknown, associates with Ro	RNA cleavage	SLE	[35]

The acronyms used in this table (and not included in the abbreviations list at the start of the review) are listed in the appendix.

may dysregulate their SRPK activity, leading to phosphorylation of serine arginine proteins [40,49]. Phosphorylated serine arginine proteins associate with both the U1-snRNP and the U3-snoRNP, which are major autoantigenic complexes in MCTD and scleroderma, respectively ([42] and our unpublished data). Although a study has not yet been performed to address whether serine arginine proteins are themselves targets of an immune response in SLE, MCTD, or scleroderma, their association with the U1-snRNP and U3-snoRNP may contribute to the immunogenicity of other components of these important autoantigen particles.

Several components of RNA polymerase I (ie S5 and S6) are specifically recognized by autoantibodies derived from SLE patients when they are phosphorylated, suggesting that phosphorylation may play a direct role in determining the immunogenicity of some proteins [50•]. Although their phosphorylation state is not known to be altered in response to stressful stimuli, RNA polymerase I components are targets of the protease granzyme B [51••]. The humoral arm may not be the only component of the immune response that is influenced by the phosphorylation state of triggering antigens. T-cell recognition of phosphopeptides bound to self-major histocompatibility complex

(MHC) molecules has also been reported for the multiple sclerosis autoantigen αB-crystallin [52]. T cells recognized and responded to preparations of αB-crystallin that differed in the extent of phosphorylation of αB-crystalline at position Ser^{45}. Interestingly, αB-crystallin is phosphorylated in glial cells after stressful stimuli, supporting the concept that T cells can specifically recognize phosphopeptides that are modified in response to stress [53].

Dephosphorylation of La and ADP-ribosylation of poly A ribose polymerase

La is the only autoantigen identified to date that undergoes dephosphorylation during apoptosis. In early studies, La was shown to be partially cleaved in response to several different apoptotic stimuli [39]. This cleavage event has been extensively characterized by Rutjes *et al* [55]. In response to a wide variety of cell death stimuli, La is partially cleaved *in vivo*, probably in its carboxyl-terminus, in a caspase-dependent manner. Somewhat surprisingly, La is also specifically dephosphorylated at Ser^{366} during apoptosis [54]. The mechanism by which this occurs is unclear and could involve inactivation of a kinase, many of which are cleaved and dysregulated during apoptosis [55]. Alternatively, activation of a phosphatase, such as protein phosphatase 2A, whose inhibitory α-subunit is cleaved and inactivated (resulting in upregulated phosphatase activity) during cell death, may also be responsible [56].

Interestingly, the enzymatic activity of poly A ribose polymerase (PARP) has recently been shown [57] to be transiently upregulated in stressed cells before their commitment to death, and several nuclear proteins (including PARP itself) are ADP-ribosylated in response to stress. This activity decreases at later time points, presumably due to inactivation of PARP upon cleavage by caspases. La and PARP are both excellent substrates for granzyme B and are cleaved at sites that generate a different pattern of proteolytic fragments than seen with caspase-mediated cell death [51••]. Which of these modifications may be involved in the generation of specific high-titer autoantibodies in SLE and related autoimmune diseases is an open question. This is further complicated for La by the observation that the Y RNAs, which exist in complex with the Ro particle, are also degraded during apoptosis [35]. It is currently unknown whether Y RNA cleavage is also observed in granzyme B-mediated cell death pathways.

Transglutamination of autoantigens

'Tissue' transglutaminase is a widely expressed enzyme that has several links with autoimmune disease. The enzyme catalyzes the cross-linking of substrate proteins through the formation of ε(γ-glutamyl) lysine cross-links and N,N-bis(γ-glutamyl) polyamine bonds. Several autoantigens are specific cross-linking substrates for tissue transglutaminase, particularly histone H2B, a component of the nucleosome discussed earlier [58]. Transglutaminase messenger RNA levels are upregulated during apoptosis [58]. The enzymatic activity of transglutaminase is believed to be activated early in the death process, but appears to be crippled at a later point through a caspase 3-mediated proteolytic event [59]. Proteolysis correlates with a loss of transglutaminase cross-linking activity. Interestingly, autoantibodies directed against transglutaminase are highly specific markers of coeliac disease, an human leukocyte antigen-DQ2-restricted inflammatory bowel disease that is triggered by exposure of the gut to wheat gliadin [60]. Although it is unknown whether transglutaminase cleavage is involved in the production of antitransglutaminase autoantibodies, the autoantibodies to transglutaminase are only part of the interesting pathogenesis of this disease. The enzymatic activity of transglutaminase has been directly implicated in the pathogenesis of coeliac disease. Transglutaminase mediates this effect through an ordered series of deamidation reactions on gliadin. The modified gliadin then binds efficiently to DQ2, which is specifically recognized by gut-derived T lymphocytes [61••]. The disease presumably is antibody independent, as patients with hypogammaglobulinemia or immunoglobulin A deficiency are not protected from development of the disease. Thus, coeliac disease represents one of the few examples discovered to date whereby a post-translational autoantigen modification directly contributes to bypassing T cell tolerance to an antigen.

Ubiquitin conjugation state of autoantigens during apoptosis

Two autoantigens undergo alterations in their state of conjugation to ubiquitin. Ubiquitinated histone H2A is present in normal cells, but is absent from cells undergoing apoptosis induced by transforming growth factor-β_1, suggesting that the ubiquitin-conjugating apparatus responsible for maintaining ubiquitinated H2A is disrupted during apoptosis [62,63]. The second antigen, topoisomerase IIα, is specifically degraded through activation of the ubiquitin proteolysis system in response to ectopic expression of adenovirus $E1A_{12S}$ [64]. Finally, ubiquitin itself has been identified as a scleroderma autoantigen, although it is not known whether the antiubiquitin antibodies are initiated in response to free ubiquitin or to ubiquitin that has been conjugated to target substrates [65].

Citrullination of autoantigens

One of the most interesting post-translational modifications that has been associated with autoimmune disease is the selective deimination of arginine to form citrulline, a reaction catalyzed by the enzyme peptidylarginine deiminase. Autoantibodies that recognize citrullinated peptides have been strongly associated with rheumatoid arthritis, with a published specificity of 96% [66••]. The antigenic source of these citrullinated peptides is unknown, and few proteins are

known to contain citrulline. These include vimentin, myelin basic protein, and several skin-associated polypeptides such as filaggrin, trichohyalin, keratin, and an unidentified 70-kDa protein. Two reports suggest that peptidylarginine deiminase may be specifically activated in response to apoptotic stressors. Asaga *et al* [67] demonstrated that vimentin is selectively deiminated when mouse peritoneal macrophages undergo apoptosis upon exposure to calcium ionophore. Mizoguchi *et al* [68] subsequently demonstrated that an unidentified 70-kDa nuclear protein is similarly modified in apoptotic rat keratinocytes. Moreover, ectopic expression of peptidylarginine deiminase in these cells reproduced the deimination of the 70-kDa protein. Identification of the relevant citrullinated autoantigen that is responsible for initiating the autoimmune response in rheumatoid arthritis would be a crucial addition to the growing understanding of this complex and fascinating disease.

Autoantigen cleavage by cytotoxic T lymphocyte granule proteases

A major problem with the modifications described above is that none of the epitopes that are produced by these enzymes should be novel or unique. All should have been 'seen' before by the immune system and should not appear foreign. Thus, if apoptosis-specific modifications contribute to autoimmune disease, they must do so by lowering the threshold for a pre-existing autoreactive lymphocyte to be activated. A much more attractive hypothesis by which tolerance might be bypassed is through the creation of truly novel epitopes that have not yet been 'seen' by the immune system. One recently discovered mechanism by which this might occur is by cytotoxic T lymphocyte (CTL)-mediated apoptosis. Natural killer cells and CTLs kill virally infected cells and tumor cells by releasing their granule constituents, which then activate target cell caspase pathways through the proteolytic activation of procaspases. In addition to procaspases, granzyme B also cleaves other host cell proteins. Casciola-Rosen *et al* [51••,69••] recently demonstrated that granzyme B uniquely cleaves a wide variety of (although not all) autoantigens (Table 1). Cleavage occurs at unique sites not recognized by caspases activated by other apoptotic stimuli such as irradiation or activation of death receptors such as Fas and the tumor necrosis factor receptor. Moreover, none of the nonautoantigenic proteins tested (eg thrombin or vinculin) were substrates for granzyme B *in vivo* or *in vitro*. Another CTL granule protease, granzyme A, cleaves the SLE autoantigen nucleolin [70]. These results strongly suggest that CTL- or natural killer cell-mediated cell death, as opposed to other forms of apoptosis, may be extremely important in the initial insult that generates novel peptide fragments that appear foreign to the organism. In addition to novel epitopes generated by granzymes, several other modified antigens have been described that are created by environmental toxins or viruses, as discussed below.

Modifications associated with nonapoptotic cell death

A hallmark of scleroderma is the production of autoantibodies that recognize components of the nucleolus. As expected, many of these nucleolar autoantigens are responsible for ribosomal assembly, particularly in the splicing of ribosomal RNA molecules. Unlike the situation for SLE, there is little evidence linking apoptosis and scleroderma. Several reports published in the past few years, however, have offered compelling evidence that cell death, particularly by necrosis or exposure of cells to the heavy metal mercury, may be important events in the genesis of scleroderma.

In 1997, Casciola-Rosen *et al* [71•] demonstrated that mercury, when added to cells in culture, specifically localized to the nucleolus. This interesting observation suggested that mercury or other toxins might somehow damage the nucleolus, setting in motion an autoimmune response to components of this organelle, and perhaps leading to multisystem autoimmune disease. Because metals are often required for oxidation reactions, these investigators asked whether scleroderma autoantigens might be damaged after mercury exposure. Several scleroderma antigens, including topoisomerase I, the large subunit of RNA polymerase II, and UBF/NOR-90 were indeed fragmented when exposed to mercury. Presumably, similar oxidation reactions occur in the vasculature of scleroderma patients, which is often characterized by episodes of hypoperfusion and reperfusion.

Not all scleroderma autoantigens were observed to be fragmented in the study of Casciola-Rosen *et al* [71•], suggesting that other modifications might also contribute to scleroderma pathogenesis. Pollard *et al* [72] had established a murine model of the immune response to the scleroderma autoantigen fibrillarin. They demonstrated that exposure of mice to mercury chloride resulted in the development of an antinucleolar autoantibody response. Production of these antibodies, which included antibodies that specifically recognized fibrillarin, was genetically restricted to the H2A region of the MHC of H-2^S mice [73,74]. They went on to demonstrate that fibrillarin is uniquely modified by mercury chloride in such a way that it is no longer recognized by antibodies [75•]. The mercury chloride-induced modification required the presence of two cysteine residues, suggesting that mercury was disrupting a disulfide bond in fibrillarin that is required for antibody recognition. Several mechanisms have been proposed to explain how mercury exposure breaks tolerance, including the following: direct activation of autoreactive T cells by binding of metal to MHC and/or peptide; and stable interaction of metal with self proteins, which then undergo selective or novel proteolysis by APCs (for review [75•]).

Exposure of cells to other environmental toxins or stressors such as ethanol, mercury chloride, hydrogen peroxide, or

heat shock results in a distinct form of nonapoptotic cell death that is caspase-independent. Autoantigens are also uniquely modified in response to these diverse cellular stressors. For example, antigens such as PARP, topoisomerase I, UBF/NOR-90, and U1-70 kD are fragmented after necrotic stimuli, and the fragments are distinct from the caspase-derived fragments observed during apoptosis [76]. Together with the studies involving mercury exposure, these reports provide *in vitro* and *in vivo* evidence that an environmental toxin may contribute to the development of a specific autoimmune response.

Modifications associated with viral infection

A number of clinical observations suggests that infection of genetically susceptible individuals with as yet unidentified virus(es) may trigger or exacerbate autoimmune diseases such as SLE. This might result from the host response to the virally-infected cell (eg CTL-mediated killing discussed above), or from disruption of normal cellular functions by virally-encoded factors. Several other mechanisms by which viral infection may be involved in the pathogenesis of autoimmune disease have also been described, albeit in a different context. First, many host proteins specifically interact with viral nucleic acid. The La protein, for example, binds to hepatitis C virus, human immunodeficiency virus, and Epstein–Barr virus RNA [77–79]. The forgotten interaction of La with Epstein–Barr virus RNAs EBER 1 and EBER 2 will almost certainly be revisited [80]. Epstein–Barr virus infection has been strongly correlated with the development of SLE in a recent report [81••]. Second, several viral proteins have been identified that interact directly and specifically with host proteins. The most intriguing examples are the herpes simplex virus proteins open reading frame (ORF)-P, unique region protein 6 (UL6), and infected cell protein (ICP)27. All three viral RNA binding proteins have been shown to interact with components of the host spliceosome, either by coprecipitation analysis (UL6 and ICP27) or in the yeast two-hybrid system (ORF-P) [82,83] (unpublished data). Both mechanisms (ie stable association of viral nucleic acid or protein with host proteins) have the potential to break tolerance to the host antigen. This could occur if the initial immune response to the viral antigen spreads to the host protein(s) in the complex by 'epitope spreading'. Alternatively, binding of the viral RNA or protein to host autoantigens might change either the conformation of the antigen or the processing of the host antigen by APCs.

The genomes of several viruses, particularly picornaviruses (eg poliovirus) and flaviviruses (eg West Nile virus) encode proteins with the potential to modify host proteins directly. For example, the poliovirus genome encodes proteases that are required for processing of viral polypeptides. Host proteins are also substrates for these proteases, and their proteolytic degradation serves an important function in the viral lifecycle by insuring that host protein synthesis is shut off while cap-independent (internally-initiated) viral protein synthesis is preferentially activated. It has recently been demonstrated that the La autoantigen is an important host substrate for poliovirus 3C protease. La is cleaved between Gln^{358} and Gly^{359}, removing a carboxyl-terminal nuclear localization motif. This prevents La from shuttling from the cytoplasm back to the nucleus [84]. Interestingly, the poliovirus 3C protease cleavage site lies in close proximity to the putative caspase cleavage site identified by Rutjes *et al* [54]. Cleavage by a viral protease at such a novel site has the potential to create neoepitopes required to break tolerance to this molecule. Other examples of autoantigens that are substrates for viral proteases include vimentin (a substrate for human immunodeficiency virus-1 protease) and histone H3 (a substrate for foot-and-mouth disease virus protease 3C) [84–86]. Substrate-specific autoantibodies have not been reported to occur in association with any of these viral infections, although it is not clear such associations have been sought.

Conclusion

Since the 'rediscovery' 6 years ago by Casciola-Rosen *et al* that apoptotic stimuli may be critically important in breaking tolerance to important autoantigens, an increasing number of death-associated autoantigen modifications have been identified [12••,38]. Although proteins that are modified during apoptosis are preferred targets of the autoantibodies found in the serum of patients with autoimmune disease, it is clear that apoptosis *per se* is not sufficient to break tolerance to these self proteins. In the adult human, millions of cells undergo apoptosis each and every hour, but most people do not develop autoimmune disease. The normal process of apoptosis, refined over evolutionary millennia, is extremely efficient and extraordinarily rapid. In most tissues, the apoptotic cell undergoes nuclear and cytoplasmic condensation, nuclear fragmentation, and clearance by neighboring parenchymal cells in less than 1 h. Because of this, the apoptotic index (ie the percentage of cells in a tissue that exhibit an apoptotic morphology) tends to be low (usually less than 1%), even in tissues such as the thymus gland in which negative and positive selection result in the apoptotic elimination of more than 90% of immigrant thymocytes. Consistent with this notion, immunization of BALB/c mice with apoptotic syngeneic cells does not result in the production of pathogenic autoantibodies that are reactive with Smith complex, Ro and La, nor is it associated with the onset of a lupus-like autoimmune disease [87•].

The problem occurs when the execution, or clearance of the apoptotic cell is delayed. This phenomenon has been demonstrated in mice lacking the first component of complement. C1q functions as an opsonin that binds to apoptotic cells and promotes their clearance by professional

APCs. In the absence of C1, the clearance of apoptotic corpses is delayed, and the apoptotic index increases in tissues such as the kidney [88]. Delayed clearance of apoptotic cells somehow increases their immunogenicity. A similar phenomenon occurs when the execution of the apoptotic program is delayed. Thus, influenza virus-induced apoptosis in macrophages has been shown to increase the immunogenicity of viral proteins [89,90]. This phenomenon requires the phagocytosis of the infected macrophage by dendritic cells. By a process of cross-priming, the dendritic cell can then present antigens derived from the infected macrophage in a highly efficient manner. Because influenza virus encodes several genes that function to inhibit apoptosis (eg NS1), virus-induced apoptosis requires many hours to complete. This delay allows the virus to replicate within the infected cell.

Because virus infection is a potent inducer of the cellular stress response, this delay also allows infected cells to induce the expression of stress response proteins such as heat shock proteins 70 and 90. These proteins are peptide-binding proteins that can function as natural adjuvants to promote the immune response to peptide antigens [91]. These proteins also function as chaperones that normally deliver peptides generated at the proteasome to the transporter in antigen processing at the endoplasmic reticulum membrane, and so are important in loading peptides onto MHC molecules. It is therefore possible that the heat shock proteins function to deliver altered self-peptides formed in apoptotic cells to the MHC complex of APCs by a mechanism of cross-priming. By this mechanism, delays in the execution of apoptosis, or the clearance of apoptotic cells, could promote autoimmunity to proteins modified during apoptosis. It will therefore be important to determine whether defects in the execution and/or clearance of apoptotic cells occurs in patients with autoimmune disease.

Another important unexplained question is how proteins expressed in every cell become targets of organ-specific autoimmune diseases. The simplest (and perhaps overly naïve) explanation for organ specificity would be if a particular apoptotic trigger (eg expression of Fas/Fas ligand, or viral infection) was inappropriately switched 'on' in the target organ. Such a mechanism might in part explain diseases such as polymyositis or type I (insulin-dependent) diabetes mellitus, both of which are characterized by impressive inflammation of the target organ and production of specific subsets of autoantibodies. Interestingly, several polymyositis-specific autoantigens (eg transfer RNA synthetases and Mi-2) are substrates for granzyme B [51••]. This raises the intriguing possibility that a muscle-trophic virus infects susceptible individuals, and that the CTL response to virally-infected cells generates neoepitopes of transfer RNA synthetases and Mi-2 to which autoantibodies are eventually produced. This concept is supported by several observations made over a decade ago, including the identification of CTLs in polymyositis biopsy specimens, and the serologic association of specific viral infections with myositis [92,93]. Similar mechanisms might underlie diseases such as multiple sclerosis and rheumatoid arthritis. Until the precise autoantigens responsible for initiating these latter diseases are identified and characterized, however, any attempts to explain the organ-specific nature of the autoantibodies would be purely speculative.

Finally, why is the autoantibody profile in patients with autoimmune diseases (particularly SLE) pleiotropic? This question has fascinated clinicians for decades. Several lines of evidence suggest that the immune response to autoantigens is driven by repeated exposure of the individual to intact 'particles' (eg spliceosomes, nucleosomes, or ribosomes; for review [94•]). Which of these particles is chosen as an immunogen may depend on the genetic background of the individual, the nature of the death stimulus, the susceptibility of individual cells to the stimulus, the packaging of autoantigen combinations within cell surface blebs, and the clearance and processing of particles by APCs described earlier. The striking take-home message of the present review is that at least one death-associated autoantigen modification, and often several modifications, affect at least one component of every major disease-specific autoantigen particle that has been identified to date (Tables 1 and 2).

It is an exciting time for all investigators who endeavor to understand better the mechanisms involved in breaking tolerance to self-antigens. If the questions posed above can be successfully answered, then the etiology of many common diseases such as rheumatoid arthritis, SLE, and type I (insulin-dependent) diabetes mellitus may be elucidated, if not solved. With this solution may come more promising disease-specific, antigen-specific, or even patient-specific therapies in the new millennium, hopefully to replace the inadequate modalities used in 20th century clinical practice.

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Appendix

Acronyms used in the tables and not listed in the abbreviations list on the first page of this review are given below:

APLA = antiphospholipid antibody syndrome;
BD = Behçet's disease;
CENP = centromere protein;
CREST = syndrome of calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangectasias;
DLE = discoid lupus erythematosus;
DM = dermatomyositis;
DNA-PK = DNA-dependent protein kinase;
ER = endoplasmic reticulum;
GA = granzyme A;
GB = granzyme B;
GVHD = graft-versus-host disease;
HIV-1 = human immunodeficiency virus 1;
hnRNP = heterogeneous ribonuclear protein;
hsp = heat shock protein;
ILD = interstitial lung disease;
MCTD = mixed connective tissue disease;
NuMA = nuclear mitotic-associated protein;
PM = polymyositis;
PM-Scl = polymyositis-scleroderma autoantigen;
Pol III = RNA polymerase III;
RA = rheumatoid arthritis;
SLE = systemic lupus erythematosus;
snoRNP = small nucleolar ribonuclear protein;
SP1 = transcription factor;
SR = serine/arginine-rich splicing factors;
SRPK = SR protein kinase;
SRP 72 = the 72-kDa component of signal recognition particle;
tRNA = transfer RNA;
U1-70 kD = the 70-kDa component of the U1-snRNP;
UBF/NOR-90 = nucleolar organizing region;
UCTD = undifferentiated connective tissue disease.

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