Apoptosis, nucleosomes, and nephritis in systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by the production of antinuclear autoantibodies. The serological hallmark of SLE is the presence of antibodies against double-stranded DNA. It is generally assumed that these anti-DNA antibodies participate in the development of lesions in this disease. The primary event inducing the formation of anti-DNA antibodies has always been puzzling, since it has been very difficult to demonstrate the presence of free DNA in serum of SLE patients [1]. Furthermore, native DNA is usually considered non-immunogenic [2, 3]. In recent years, several studies have been published which suggest that not DNA itself, but DNA complexed to histones (nucleosomes) is the immunogenic particle involved both in the induction of pathogenic anti-DNA antibodies, and in the pathophysiology of SLE.

In eukaryotic cells, DNA is packed in the nucleus in the form of chromatin. In chromatin, adjacent nucleosomes are linked by about 60 bp of DNA. Nucleosomes are the fundamental repeating units of the chromatin string, and are responsible for the compaction of DNA. Each nucleosome is built up by eight core histone molecules (forming the histone octamer), one histone H1 molecule and 146 bp of DNA [4], as shown in Figure 1.

Lately, it has become evident that at least in certain murine models of SLE (lpr and gld mice), and perhaps also in SLE patients as well, the process of apoptosis is aberrant. Apoptosis (programmed cell death) results in fragmentation of the nucleus by internucleosomal cleavage of the chromatin. Normally, these apoptotic cells are phagocytosed rapidly, but if this does not occur nucleosomes may be released. On the other hand, apoptosis of maturing T cells is involved in establishing and maintaining tolerance, and therefore a disturbed apoptosis may cause a breakdown of tolerance. These recent data provide elements for a new concept of SLE, both for the induction as well as for the effector phase of the disease. We will first review the evidence for disturbed apoptosis in SLE, and then discuss how this relates to the immune response against nucleosomes. Subsequently, the significance of nucleosomes for the disease manifestations of SLE (with emphasis on glomerulonephritis) will be discussed.

Dysregulation of apoptosis in SLE

The term “apoptosis” was first used in 1972 to describe the process in which cells undergo several morphological changes, including condensation and fragmentation of the nucleus without disruption of the plasma membrane [5]. It was later shown that activation of an endogenous nuclease is involved in the internucleosomal cleavage of chromatin [6]. The induction of apoptosis is under strict regulation by a variety of factors, including signals from surrounding cells. With respect to T cells, for example, both cytokines (such as interleukin-1, interleukin-2, interferon-γ) and costimulatory molecules (like ICAM-1, VCAM-1, LFA-3 and B7) present on antigen-presenting cells determine whether a T cell is driven to apoptosis or to cell proliferation [7-10]. Once a cell has become apoptotic, it should be removed in a rapid and efficient manner while still intact, to prevent the release of potentially toxic cell contents. This removal of apoptotic cells occurs by phagocytosis. Macrophages are the most important effector cell in this respect, but other cell types like epithelial cells apparently can also ingest neighboring cells undergoing apoptosis. Several different receptors on the phagocyte surface may be involved in this process, such as phosphatidylserine receptor and thrombospondin receptors [11]. The morphology of apoptotic cells in tissues and in vitro has been described in detail [12, 13].

It has been very difficult to demonstrate the presence of free DNA in serum from SLE patients, but when DNA was found it occurred in the form of (oligo)nucleosomes [1]. A potential source for these nucleosomes are apoptotic cells, since nucleosomes were spontaneously released from cultured normal murine spleen cells, whereas this process did not occur when apoptosis was blocked with zinc sulfate [14]. Also during culture of human lymphocytes nucleosome release occurred, which was strongly correlated with the degree of lymphocyte apoptosis [15]. It is, however, not precisely known how nucleosomes are released from apoptotic cells. It is conceivable that in some cases these nucleosomes derive from apoptotic bodies. These appear at the cell surface of several cell types during apoptosis as a result of packaging of cellular components as membrane-bounded blebs. After their budding from the cell, apoptotic bodies are usually rapidly ingested by phagocytes. Some of these apoptotic bodies contain chromatin [13]. In a recent study on UV-injured keratinocytes, it was demonstrated that some of the apoptotic bodies contained nucleosomes, ribonucleoproteins (that are also targets of autoantibodies in SLE), and in addition fragments of the endoplasmatic reticulum or the nuclear membrane [16]. Nucleosomes may, after their release from apoptotic cells, also adhere to specific “nucleosome receptors” present on the surface of viable cells [17, 18]. In contrast to the programmed process of apoptosis, cell death by necrosis is a more random process of cellular destruction [12, 13] that is not expected to yield intact nucleosomal particles.

In recent years it has become evident that, at least in certain murine models, SLE is directly linked to a defect in apoptosis. We will first discuss the data obtained in lupus-prone and transgenic mice, and then address this question for human SLE. In mice, the lpr and gld genes provide single gene models of systemic autoimmunity (including the formation of anti-dsDNA antibodies).
Although expression of the lpr gene in both B and T cells is required for autoantibody production in lpr mice [19, 20], the molecular basis of the lpr syndrome became only apparent when Watanabe-Fukunaga et al [21] demonstrated that lpr mice lack the expression of the Fas antigen. The Fas molecule (also known as APO-1, and designated CD95) belongs to the TNF receptor family, and is a cell-surface receptor that mediates apoptosis. The aberrant transcription of the Fas gene in lpr mice is caused by the insertion of a retroviral sequence in an intron [22-24]. Early replacement of the lpr gene by the normal Fas gene will correct the accelerated autoimmune disease in these mice [25]. These findings demonstrate that in this model there is a causal relationship between dysregulation of apoptosis and autoimmune disease. The natural ligand for Fas has recently been cloned; it is a transmembrane protein and a member of the TNF family [26]. The gld mouse strain has a mutation of the Fas ligand gene [27]. It therefore appears that either a defect in the receptor (lpr) or its ligand (gld) causes systemic autoimmunity. How does the defect in the Fas antigen cause autoimmunity in lpr mice? In view of the important role of apoptosis in shaping the T cell repertoire by negative selection, one is inclined to think that a defective Fas antigen would cause aberrant thymic selection. It appears, however, that thymic selection of CD4+ or CD8+ T cells in these mice proceeds normally [28]. In contrast, both subsets of mature T cells exhibit decreased apoptosis in response to antigen stimulation and therefore it has been suggested that autoimmunity in this model results from a failure of peripheral tolerance [29, 30]. Also in gld mice, the T cell defect apparently is contained within the compartment of activated, mature T cells rather than in the thymus [31]. It seems probable that the defective Fas gene in lpr mice may also cause a decreased antigen-innuced apoptosis of mature autoimmune B cells [21]. It is important, however, to realize that even in lpr mice other genes are involved as well [32]. Since MRL+/+ mice (which lack the lpr gene) also develop lupus (although to a lesser extent, and later in life), it must be concluded that lpr accelerates rather than causes the disease. In other murine models of SLE, such as NZB/W mice, no abnormalities of the Fas gene have been detected [22].

Apart from the Fas gene, there are several other genes involved in the regulation of apoptosis. There are pathways for the induction of apoptosis which are independent of the Fas antigen, such as the TNF receptor [33]. Furthermore, there are genes that decrease the susceptibility for apoptosis, such as the bcl-2 gene [34]. The bcl-2 gene was first discovered in B cell lymphomas, which have a permanent high expression of the bcl-2 protein. When in transgenic mice bcl-2 was brought under the control of an immunoglobulin promoter, the number of B cells increased, and autoantibodies against nuclear antigens (including histone and DNA) were produced, resulting in immune complex glomerulonephritis [35]. In another study with transgenic mice, it could be demonstrated that bcl-2 inhibits the clonal deletion of autoreactive B cells in the periphery but not in bone marrow [36]. In bcl-2 knockout mice, fulminant lymphoid apoptosis was observed, causing disappearance of the lymphoid system within four weeks after birth [37, 38]. Recent studies have revealed that the bcl-2 gene is a member of an expanding family of similar genes, some of which have the same effect as bcl-2 (protection against apoptosis), whereas others like bax will increase the susceptibility for apoptosis [39]. The bax protein can form a heterodimer with the bcl-2 protein and in this way neutralize the effect of bcl-2. The bcl-2 protein apparently exerts its protective effect by activating an anti-oxidant pathway in the cell [40]. Many stimuli inducing apoptosis are known to generate reactive oxygen species. The relative amounts of bax and bcl-2 in a cell may therefore determine the susceptibility of the cell for apoptosis.

In human SLE, no defects in expression or function of the Fas antigen have been found [41]. This does not imply, however, that apoptosis is completely normal in human SLE. Two phenomena have been described that may contribute to disturbed apoptosis in human SLE. First, serum levels of soluble Fas were found to be elevated in several SLE patients. Due to alternative splicing, the Fas encoded protein can also occur in a soluble form. Soluble Fas can inhibit apoptosis, since after injection in normal mice, it causes increased numbers of B cells and autoimmune features [42]. Secondly, in lymphocytes from SLE patients the expression of bcl-2 is increased [43]. This may be a reflection of in vivo activation, since lymphocyte activation is known to increase the transcription of bcl-2 [44]. Despite these increases of soluble Fas and intracellular bcl-2, lymphocytes from SLE patients display an increased rate of “spontaneous” apoptosis in vitro [15], as do lymphocytes from lpr mice [45]. One should be cautious, however, to extrapolate from these in vitro findings to the in vivo situation.

**Fig. 1**. Diagram showing how the nucleosome is built up by eight histone proteins (4 homodimers of H2A, H2B, H3, and H4) and two superhelical turns of 146 bp of DNA. The N-terminal parts of the histone molecules (approximately one third of the total molecule) contain positively charged residues which are located on the outside of the nucleosome. Histone H1, which is located on the outside of the nucleosome, is not shown in this diagram.
It has been postulated that lymphocytes from lpr mice may be "primed" for apoptosis, but prevented from dying in vivo. Once placed in culture in vitro, such cells would then rapidly die [45]. With respect to lymphocytes from SLE patients, the protective effect of soluble Fas in serum will be diminished or absent when cells are cultured in vitro. Furthermore, as mentioned above, the rate of apoptosis of SLE lymphocytes in response to antigenic stimulation is decreased [29, 30] instead of increased. It may be important in this respect to precisely define the subset of lymphocytes that is being studied (double-negative T cells, or CD4-positive T cells, or B cells), and their antigen specificity. It is conceivable that some cells in SLE patients (or lupus-prone mice) display an increased rate of apoptosis whereas for other cells (depending on cell type, activation state, and specificity) apoptosis may proceed at a decreased rate, or at the same rate as in normal individuals. Therefore, although the Fas antigen is normal in human SLE, apoptosis appears to be abnormal, as evidenced by an increased concentration of soluble Fas, an increased expression of bcI-2, and an increased rate of apoptosis when lymphocytes from SLE patients are cultured in vitro. It remains to be proven that the abnormalities of apoptosis observed in human SLE are a cause of disease (as appears to be the case in the murine SLE models discussed above) rather than a consequence.

Dysregulation of apoptosis can contribute to the pathogenesis of lupus nephritis in two different ways. First, apoptosis appears to be an important mechanism to induce tolerance of T cells towards self-antigens [46, 47]. This implies that a disturbance in apoptosis may cause persistence of autoreactive T cells and in this way affect the magnitude of the anti-nucleosomal immune response. As discussed above, one of the consequences of a defective Fas antigen is breakdown of peripheral T cell tolerance. Secondly, autoreactive lymphocytes that have escaped apoptosis in response to autoantigen may be "primed" for apoptosis. Some of these cells may be induced to become apoptotic by other mechanisms (unrelated to Fas). Removal of such apoptotic lymphocytes may be less effective (depending on the microenvironment) than when apoptosis occurs by the "normal" (antigen-induced) mechanism. In this respect it may be relevant that within tissues phagocytosis of apoptotic bodies occurs rapidly, but not when they are formed in a fluid medium [13]. A possibility that deserves further investigation is that in SLE patients phagocytosis of apoptotic cells (and apoptotic bodies) might be decreased. Although there are no published data yet on this subject, it has been known for a long time that in SLE there are profound defects in other forms of phagocytosis (such as complement-mediated phagocytosis in lupus-prone mice [48], and Fc receptor-dependent phagocytosis in SLE patients [49]). If phagocytosis of apoptotic cells is indeed defective in SLE, this could also affect the persistence of non-lymphoid apoptotic cells (such as UV-injured keratinocytes [16]).

Nucleosomes as immunogenic particles in SLE

In what way can dysregulation of apoptosis contribute to autoimmunity against nucleosomal antigens in SLE? What is the role of nucleosomes, and how are T lymphocytes (double-negative, or CD4+ cells) and B cells involved in this process? As discussed above, apoptosis is the most likely source of nucleosomes. Lymphocytes from SLE patients demonstrated an increased rate of apoptosis in vitro, and the number of apoptotic cells in freshly isolated lymphocytes was also slightly increased [15]. If this reflects the in vivo situation, the increased number of apoptotic lymphocytes might lead to the release of increased amounts of nucleosomes. One way that nucleosomes can evoke an immune response is by polyclonal activation of B cells. Both with murine and human lymphocytes, polyclonal B cell activation by nucleosomes has been demonstrated [14, 50]. The secretion of interleukin-6 that was observed after interaction of nucleosomes with murine spleen cells [18] may further enhance this polyclonal B cell activation.

Although polyclonal B cell activation by nucleosomes may occur, there are many arguments in favor of the hypothesis that pathogenic anti-DNA antibodies are the result of an antigen-driven immune response (such as clonal expansion, IgG class switch, somatic mutations). T-helper (Th) cells are required for the production of these anti-DNA autoantibodies. Since there is an expanded population of double-negative T cells (CD3+, CD4+, CD8-) in SLE [51, 52], and because these cells exhibit a preferential use of distinct Vβ genes both in lpr mice [51] and in humans [53], these cells might be involved in the initiation of autoimmune disease. Indeed, aβ CD4−CD8− (as well as γδ CD4−CD8−) T helper cell lines could be isolated from patients with active lupus nephritis [52, 54]. The large majority, however, of Th cells supplying help for production of pathogenic anti-DNA, are CD4+ cells and not double-negative cells [54]. Furthermore, recent experiments with MHC class II-deficient lpr mice have demonstrated an important role for CD4+ Th cells, whereas double-negative T cells did not appear to be essential for initiating disease [55]. It was found in lupus-prone mice that only a fraction of autoreactive T cell clones can induce anti-DNA autoantibodies in vitro, and accelerate the development of lupus nephritis when transferred in vivo. The specificity of these pathogenic Th cells has been studied in some detail. Approximately 50% of them were specific for nucleosomal antigens. Importantly, nucleosome-specific CD4+ T cells were not detectable in normal mice, but they were found in the spleens of lupus-prone mice as early as one month of age, long before any autoimmune manifestation. These nucleosome-specific Th cells not only augmented the anti-nucleosome response of syngeneic B cells, but also the anti-histone and anti-DNA response. The importance of nucleosomes as immunogenic particles was underlined by the finding that injection of purified nucleosomes into preautoimmune mice could significantly accelerate the development of glomerulonephritis [56].

An intriguing question in this respect is the nature of the epitope recognized by the pathogenic Th cell. These nucleosome-specific Th clones were not activated by free DNA or histones, the two components of nucleosomes. This result suggests that either nucleosomes are taken up more efficiently by antigen-presenting cells, or that critical Th cell epitopes in the histones are protected from degradation by being bound to DNA [56]. Another possibility is that after antigen processing of nucleosomes, MHC class II molecules present self-peptides that were previously cryptic. The possible significance of such cryptic epitopes for the induction of autoimmunity has recently been reviewed [57]. It has also been suggested that the presence of endoplasmatic reticulum or nuclear membrane within the apoptotic bodies causes an increased generation of reactive oxygen species which may induce oxidative modification of autoantigens. The unique peptide fragments generated in this way could then, in genetically susceptible individuals, be presented to Th cells and induce an autoimmune response [16].

Whatever the precise nature of the epitope recognized by the
pathogenic Th cells, it is obvious that nucleosome-specific Th cells were only present in lupus-prone and not in normal mice [56]. In normal individuals, apoptosis also occurs but no anti-DNA antibodies are found. Furthermore, injection of nucleosomes into normal mice does not result in formation of anti-DNA autoantibodies or nephritis [58]. It is apparent that apart from the extracellular presence of nuclear antigens, there must be other factors involved in the induction of such autoimmune T cells. It is well known that susceptibility to SLE is influenced by genes encoded within the MHC complex [2, 55]. Furthermore, in lpr mice the virtually absent expression of Fas may cause breakdown of (peripheral) tolerance, as discussed above. This may result in the prolonged survival in vivo of autoimmune T and B cells.

**Anti-nucleosome antibodies**

The occurrence of antibodies specific for nucleosomes or subnucleosomal structures in SLE provides further evidence that nucleosomes are immunogenic particles in SLE. These antibodies, which only react with the intact nucleosome or subnucleosomal structures, but not with its components DNA or individual histones, have first been noted in drug induced lupus. In procainamide induced lupus not only antibodies to H2A-H2B complex were found [59], but also to (H2A-H2B)DNA complexes [60]. Further analysis revealed that the antibodies recognize an epitope created by the monomeric, trimolecular histone-DNA complex [61]. Studies in lupus prone mice revealed that antibodies against chromatin (such as nucleosomes) are the result of an antigen driven response and precede the occurrence of anti-DNA or anti-histone antibodies [62]. The authors concluded that anti-DNA antibodies are a subset of the wide spectrum of anti-

chromatin (nucleosome) antibodies, which is also favored by the observation that nucleosome specific T helper cells cannot only induce an anti-nucleosome response, but also reactivity towards DNA [56]. Recently, it was shown that the prevalence of anti-nucleosome antibodies is very high (88%) in human lupus [63] and that the titers of anti-nucleosome reactivity correlate with disease severity [64]. One should realize, however, that the study of these reactivities in polyclonal SLE sera is hampered by the fact that anti-DNA and anti-histone antibodies will also bind to nucleosomes. This problem is circumvented if one studies the specificity of monoclonal antibodies derived from lupus prone mice or SLE patients. Indeed, reactivity against histone/DNA complexes has been found in such monoclonal antibodies (mAbs). From MRL/Mp ++ mice mAbs were obtained which exerted reactivity against the (H2A-H2B)DNA complex [65], and recently a murine mAb directed against the intact nucleosome, which did not recognize any subnucleosomal structure, was described [66]. Also in mAbs derived from SLE patients reactivity against a mixture of DNA and histones has been detected [67]. We recently found that anti-nucleosome antibodies complexed to nucleosomal material exerted anti-DNA reactivity both in ELISA and Farr assay. After removal of the bound nucleosomal material the anti-DNA reactivity disappeared and only anti-nucleosome reactivity remained [68]. This indicates that in SLE sera reactivity towards DNA can be a feature of anti-nucleosome antibodies. In fact, we now have preliminary data that after purification of anti-DNA positive SLE sera, a substantial part of the anti-DNA reactivity is lost, whereas reactivity against other, nominal antigens (for instance anti-EBV) is unaltered. This suggests that the occurrence of anti-nucleosome antibodies in SLE might be more prevalent than is assumed until now.

**Relevance of nucleosomes for the pathophysiology of SLE nephritis**

Classically, not nucleosomes but DNA has been thought to play a role in the development of tissue lesions in lupus through the formation and subsequent deposition of DNA/anti-DNA immune-complexes [69]. However, formal proof for this assumption is rather lacking. It has been very difficult to detect circulating free DNA or DNA complexed to anti-DNA antibodies [70–72]. Rumore and Steinman [1] showed that DNA in the circulation of SLE patients was present in the form of (oligo)nucleosomes. Based on these observations it is likely that DNA is not present in naked form in the circulation but complexed in (oligo)nucleosomes. In addition, it is difficult to envisage how the anionic charged DNA can have affinity for the anionic charged GBM. The first notion, that nucleosomes might play a role in the pathophysiology of glomerular disease in lupus, came from experiments with cross reactive anti-DNA antibodies. Working with anti-DNA mAbs which bound to heparan sulfate (HS) we found that this cross reactivity was not exerted by the antibody itself, but was mediated by nucleosomal material complexed to the antibody [73]. HS is an intrinsic constituent of the GBM, and the negatively charged side chain of heparan sulfate proteoglycan. It is responsible for the negative charge of the GBM, and thus for the charge-dependent perselectivity of the GBM [74]. Neutralization of this HS associated charge, or antibody binding to HS leads to albuminuria [75, 76]. We postulated that the positively charged histones within the nucleosomal part of the immune complex
could interact with HS and that this interaction might lead to albuminuria by neutralization of charge or by eliciting an inflammatory reaction [73]. At the same time Schmiedeke et al [77] indeed showed that histone aggregates have a high affinity for the GBM. We subsequently showed in a rat kidney perfusion system that histones can mediate binding of subsequent perfused DNA and anti-DNA and induce subepithelial or subendothelial localization of the antibody depending on the perfusion protocol [78]. We propose that nucleosomes act as planted antigens or as mediators for binding of autoantibodies to the GBM. Although nucleosomes have a pi of about 7, they contain many positively charged N-terminal regions which are located on the outside of the particle. It has even been shown that molecules with a net negative charge, but with cationic regions can bind to the GBM [79]. Indeed, we could show that nucleosome containing immune complexes are able to bind to the GBM, whereas the purified antibodies do not bind. Binding of such complexes to the GBM is illustrated at the ultrastructural level in Figure 2. The pathogenic potential of this binding was underlined by the fact that complement activation took place. Using heparinase perfusion prior to perfusion of nucleosome containing immune complexes, we could show that the ligand in the GBM was partly but not solely HS [68]. The exact composition of the nucleosomal material in the immune complex which mediates the binding to the GBM in vivo is not clear yet. In our studies we obtained immune complexes from hybridoma culture supernatants and found that the four core histones but not H1 were present, and DNA in the complexes were predominantly the size of about 120 bp. The presence of antibody bound to the nucleosome seems to be a critical determinant, since we found that perfusion of naked nucleosomes did not lead to a comparable binding to the GBM. Furthermore, the specificity of the bound antibody could be important for the overall pi of the complex, for instance anti-DNA and anti-nucleosome antibodies increase the pi of the complex and thereby the nephritogenicity. This concept is supported by the recent finding that pathogenic anti-DNA antibodies, which are able to bind to HS in the GBM via nucleosomes, harbor more charged amino acids (like arginine) than non-pathogenic anti-DNA antibodies [80]. In this concept binding of anti-histone antibodies, by binding to the histone part of the nucleosome would both decrease the pi and the nephritogenicity of the complex. This latter hypothesis is in line with the clinical observation that renal disease is rare in SLE-like syndromes characterized by the occurrence of predominantly anti-histone antibodies as in drug induced lupus.

What are the data to support the significance of nucleosomes in vivo? Since HS reactivity is a feature of immune complexes consisting of anti-nuclear autoantibodies and nucleosomal antigens, anti-HS reactivity in plasma is an indirect way to identify the presence of these immune complexes. Anti-HS reactivity has been found to correlate with renal symptoms in lupus patients [81–83], although anti-HS reactivity was not present in every episode with renal manifestations [83]. Further evidence for the role of these immune complexes comes from our observation that the staining for glomerular HS in both human [84] and murine [85] lupus nephritis is strongly reduced or even absent. Despite a nearly complete loss of HS staining in mice with heavy and prolonged albuminuria, glomerular HS content was unaltered. Therefore, we had to conclude that HS was masked by immunoreactants presumably containing nucleosomal antigens. More direct evidence for the involvement of nucleosomes in lupus nephritis is provided by studies showing both histones [86, 87] and DNA [88] in glomerular immune deposits in human and murine lupus nephritis. In one study the deposition of histones was almost exclusively found in albuminuric mice [87], underlining the role of histones and nucleosomes in the development of albuminuria in these mice. These in vivo data therefore support the concept that nucleosomal material may mediate antibody binding to the GBM for which HS is a ligand.

In conclusion, both in human and murine lupus abnormalities in apoptosis have been described. This aberrant apoptosis may contribute to lupus nephritis by mechanisms that have been summarized in Figure 3. Binding of nucleosome containing complexes to other basement membranes might lead to other organ manifestations of the disease. However, many questions remain to be answered. Future studies have to clarify the exact contribution of disturbed apoptosis in SLE. In addition studies are needed to identify the precise nature of the epitopes within the nucleosome recognized by auto-reactive T cells. Furthermore, the possibility should be studied that different epitopes within the nucleosome
dictate the production of different autoantibodies (such as anti-histone, anti-DNA, anti-nucleosome). Finally the exact composition of the nucleosome and the nucleosome/autoantibody complex determining affinity for the GBM should be clarified.

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Note added in proof

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