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Lack of 'tissue' transglutaminase protein cross-linking leads to leakage of macromolecules from dying cells: relationship to development of autoimmunity in MRL*lpr/lpr* mice

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Abstract

Genetic defects of the CD95 (Fas/Apo-1) receptor/ligand system, has recently been involved in the development of human and murine autoimmunity. We investigated whether a deregulation of the 'tissue' transglutaminase (tTG), a multifunctional enzyme which is part of the molecular program of apoptosis, may act as a cofactor in the development of autoimmunity. We found that MRL*lpr/lpr*, which are characterized by a defect in the CD95 receptor and suffer of a severe systemic lupus erythematosus-like disease, produce large amounts of circulating tTG autoantibodies. This phenomenon is paralleled by an abnormal accumulation of an inactive enzyme protein in the accessory cells of lymphoid organs. To investigate the molecular mechanisms by which tTG inhibition may contribute to the development of autoimmunity we generated a cell culture model system consisting of L929 cells stably transfected with a full length tTG cDNA. When L929 cells were killed by Tumor Necrosis Factor α (TNFα) a pronounced release of DNA and Lactate Dehydrogenase (LDH) was observed. Overexpression of tTG in these cells largely prevented the leakage of macromolecules determined by TNFα treatment, an effect which is abolished by inactivating the enzyme cross-linking activity by a synthetic inhibitor. These *in vitro* observations provided the basis to explain the increased levels of plasmatic LDH we detected in MRL*lpr/lpr* mice. These data suggest that lack of an active tTG may represent a cofactor in the development of autoimmunity.

Keywords: apoptosis, follicular dendritic cells, lymph nodes, autoantibody, LDH, TNFα

Abbreviations: tTG, 'tissue' transglutaminase; TNFα, tumor necrosis factor α; LDH, lactate dehydrogenase; HSP, heat shock protein; LC1, lipocortin-1; APC, antigen presenting cell; PLC, phospholipase-C; PBS, phosphate buffer saline; SSC, standard saline citrate; *lpr*, lymphoproliferative gene.

Introduction

'Tissue' transglutaminase (tTG; E.C. 2.3.2.13) is a multifunctional protein which, in its protein cross-linking configuration, catalyzes a Ca^{2+} and thiol-dependent acyl-transfer reaction among polypeptide chains, forming $\varepsilon(\gamma$-glutamyl)lysine and N,N-bis(γ-glutamyl)polyamine isodipeptide linkages (Folk, 1980; Fesus *et al*, 1991; Piacentini *et al*, 1994). The formation of these covalent cross-links leads to protein polymerization conferring resistance to physical stresses as well as insolubility (in SDS and chaotropic agents) to the polypeptides included into the polymer (Folk, 1980; Fesus *et al*, 1989, 1991; Piacentini *et al*, 1991, 1994).

Several laboratories have demonstrated the association between apoptosis and the induction and activation of tTG both *in vivo* and *in vitro* (see Fesus *et al*, 1991; Piacentini *et al*, 1994 for review). The covalent bonds formed by the tTG-catalyzed reactions in the apoptotic bodies are irreversible, in fact, endoproteases capable of hydrolyzing these cross-links have yet to be identified in vertebrates (Fesus *et al*, 1991; Piacentini *et al*, 1994). As a consequence free $\varepsilon(\gamma$-glutamyl)lysine, the degradation product of the tTG crosslinked polymers present in apoptotic bodies, is detectable both in the culture fluid and in plasma (Fesus *et al*, 1991; Harsalvi *et al*, 1992). Evidence has also been presented indicating that tTG might be involved in killing (Piacentini, 1995; Melino *et al*, 1994; Gentile *et al*, 1992; Wyllie *et al*, 1980).

One important feature distinguishing apoptosis from necrosis *in vivo* is that in the former there is an efficient disposal of apoptotic bodies which prevents both the inflammatory response and the exposure of self antigens which may lead to the development of autoimmunity (White, 1996).

MRL/Mp mice with a lymphoproliferative gene, *lpr* (MRL/Mp-*lpr/lpr*), develop severe autoimmune diseases such as lymphadenopathy, glomerulonephritis, arteritis and arthritis associated with an age-dependent massive production of autoantibodies which determine their premature death (Cohen and Eisenberg, 1991; Theofilopoulos, 1995; Nagata and Golstein, 1995). It has been demonstrated that the lymphoproliferation disorder described in the *lpr*

mice is characterized by a defect in the CD95 antigen that mediates apoptosis (Nagata and Golstein, 1995; Watanabe-Fukunaga, 1992). However, the pathogenesis of these autoimmune diseases cannot be explained by the lack of CD95-mediated apoptosis alone; the dysfunction of other gene products, particularly of those involved in the disposal mechanisms of apoptosis has also been hypothesized (Theofilopoulos, 1995). This study was undertaken to verify whether an impairment of tTG-catalyzed covalent polymerization of proteins in dying cells leads to the release of intracellular components and whether this phenomenon is related to the development of autoimmune syndromes such as that affecting MRL*lpr/lpr* mice.

Results

Presence of autoantibodies against apoptosis-related gene products in MRL*lpr/lpr* mice

MRL*lpr/lpr* mice show an age-dependent increase in autoantibodies against the oligonucleosomes fragments produced by the activation of endonuclease(s) during apoptosis which is associated with the overproduction of several cytokines including TNFα (Cohen and Eisenberg, 1991, Theofilopoulos, 1995, Nagata and Golstein, 1995; Yokoyama *et al*, 1995; Tang *et al*, 1991; Levine *et al*, 1991; Izui *et al*, 1984). To verify whether additional abnormalities related to the cell death programme may have a role in the development of autoimmunity in MRL*lpr/lpr* mice, we first investigated the presence of serum autoantibodies against proteins and intracellular macromolecules produced during apoptosis.

Figure 1 shows that 30 week-old MRL*lpr/lpr* mice have high levels of anti-tTG and 70 kDa Heat Shock Protein (HSP) IgGs whose synthesis is elevated in cells undergoing apoptosis (Buttyan *et al*, 1988).

Accumulation of an inactive tTG in the lymphoid organs of aging MRL*lpr/lpr* mice

In an attempt to investigate the abnormal production of anti-tTG antibody the expression of tTG in the lymph nodes and thymus of young (7 weeks) and old (30 weeks) MRL+/

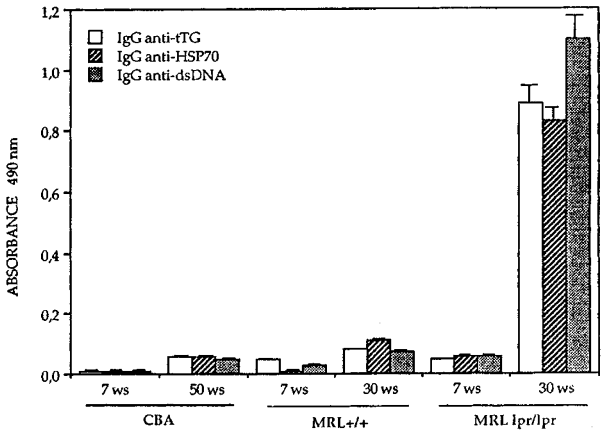

Figure 1 Presence of antibodies to tTG, HSP70 and dsDNA in MRL*lpr/lpr* mice. High levels of IgGs were detected only in the sera of 30-week-old MRL*lpr/lpr* mice. ELISA assay was performed as described in Materials and Methods. Data were expressed as the mean $\pm$ standard error (S.E.M.) of four determinations for each experimental group.

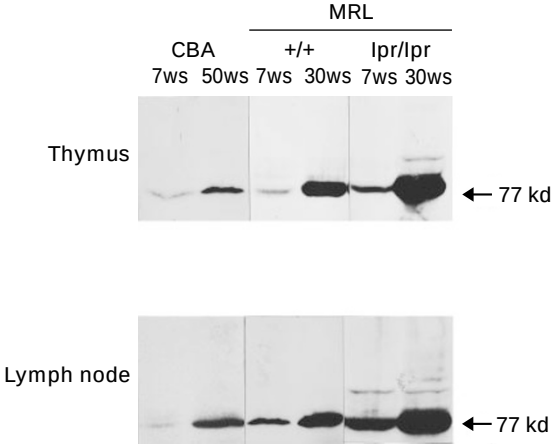

Figure 2 Effect of aging on tTG protein levels in thymuses and lymph nodes from CBA, MRL+/+ and MRL*lpr/lpr* mice. Thymuses and lymph nodes from CBA, MRL+/+ and MRL*lpr/lpr* mice at different ages were homogenized and Western blot analysis was carried out on aliquots (100 μg) of the total protein extracts as described in Materials and Methods.

Table 1 Effect of aging on 'tissue' transglutaminase activity in thymus and lymph node of CBA, MRL+/+ and MRL*lpr/lpr* mice

	Age	Thymus			Lymph Node		
	(weeks)	A	B	A/B*	A	B	A/B
CBA							
	7	0.5 $\pm$ 0.1	1.0 $\pm$ 0.3	(0.5)	0.7 $\pm$ 0.4	1.2 $\pm$ 0.2	(0.6)
	50	0.8 $\pm$ 0.2	1.6 $\pm$ 0.3	(0.5)	1.5 $\pm$ 0.5	3.0 $\pm$ 0.5	(0.5)
	7	0.6 $\pm$ 0.1	1.0 $\pm$ 0.3	(0.6)	0.5 $\pm$ 0.2	1.0 $\pm$ 0.2	(0.5)
MRL+/+							
	30	2.3 $\pm$ 0.7	3.8 $\pm$ 0.7	(0.6)°	2.6 $\pm$ 0.8	6.5 $\pm$ 1.2	(0.4)°
	7	0.7 $\pm$ 0.1	2.3 $\pm$ 0.3	(0.3)°	1.0 $\pm$ 0.3	5.0 $\pm$ 0.9	(0.2)°
MRL*lpr/lpr*							
	30	2.3 $\pm$ 0.5	11.5 $\pm$ 1.3	(0.2)°	2.7 $\pm$ 0.5	13.5 $\pm$ 0.2	(0.2)°

(A) tTG enzymatic activity expressed as nmol/h/mg protein (B) tTG protein levels evaluated by densitometric analysis of Western blot analyses as reported in Figure 2 and expressed as arbitrary units. (*) The relative specific activity is reported in parenthesis, values have been calculated by dividing the above reported tTG enzymatic activities (A) by the respective amount of enzyme protein (B). Data are the mean of triplcate determinations with a S.E.M. in four different experiments. (°) Statistically significant ($P < 0.01$) with respect to the relative CBA controls.

+ and MRL*lpr/lpr* as well as of control mice (7 and 50 weeks old CBA mice) was analyzed. Figure 2 shows that the tTG gene is highly expressed in the thymus and lymph nodes both from MRL+/+ and MRL*lpr/lpr* as compared to control CBA mice. While a slight age-dependent increase was noticed in 50-week-old CBA mice, an abnormal accumulation of the tTG protein was detected both in the thymus and lympth nodes of 7-weeks and 30-week-old MRL*lpr/lpr* mice (Figure 2). However, the increase in the enzyme activity detected in MRL*lpr/lpr* mice (Table 1) does not match the enzyme protein level observed in the lymphoid tissues of the same animals (Figure 2). In fact, a significant early decrease in the relative specific activity of tTG (activity/antigen, Table 1) was observed in MRL*lpr/lpr* mice compared to that observed in MRL+/+ and CBA mice, suggesting that an inactive tTG is present in MRL*lpr/lpr* mice. This hypothesis has been confirmed *in vivo* by measuring of tTG-dependent formation of the SDS-insoluble cross-linked polymers in lymphoid cells from MRL*lpr/lpr* mice at different ages and the accumulation in plasma of MRL*lpr/lpr* mice of free $\varepsilon(\gamma$-glutamyl)lysine (Fesus *et al*, 1991). Data reported in Figure 3 show that the age-dependent accumulation of tTG protein in cells extracted from the lymph nodes of MRL*lpr/lpr* mice was not paralleled by a proportional increase in the number of cross-linked protein scaffolds. In addition, no significative change in the plasma level ($1-2$ μmol/L) of $\varepsilon(\gamma$-glutamyl)lysine, which reflects the overall cross-linking activity in the body (Fesus *et al*, 1991; Harsalvi *et al*, 1992), was observed in MRL*lpr/lpr* when compared to CBA and MRL+/+ mice (data not shown). Taken together these data suggest that an inactive tTG is present in MRL*lpr/lpr* mice.

To determine whether the discrepancy observed between the accumulation of enzyme protein and its activity was associated with a rearrangement of the tTG gene in MRL*lpr/lpr* mice, Southern analysis of genomic DNA from CBA, MRL+/+ and MRL*lpr/lpr* mice was carried out using a mouse tTG cDNA probe. The Southern analysis of genomic DNA from all animals analyzed did not show extra bands in BamHI, EcoRI and pStI digested DNAs indicating that there is no rearrangement of the tTG gene (data not shown).

To determine in which cells the abnormal accumulation of tTG protein observed in *lpr* mice takes place, the localization of the enzyme protein in the thymuses and lymph nodes obtained from young and old CBA, MRL+/+ and MRL*lpr/lpr* mice was analyzed. Figure 4 shows that accumulation of tTG protein in autoimmune mice occurred mainly in the stromal cells which form the basic architecture of lymphoid organs (Ibrahim *et al*, 1995).

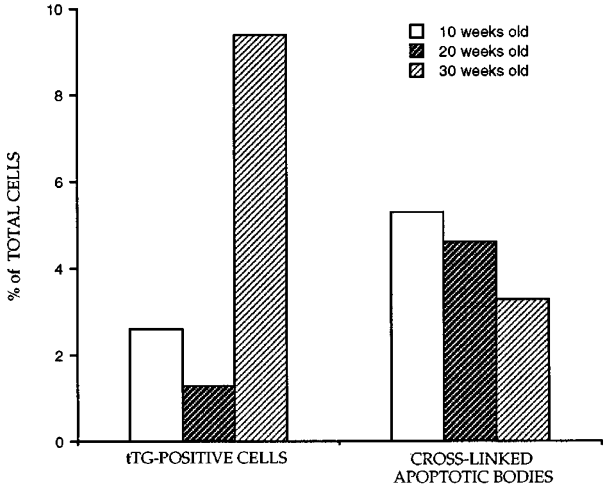

Figure 3 Evaluation of cross-linked apoptotic bodies in MRL*lpr/lpr* mice. The percentage of apoptotic cells was evaluated by counting the tTG positive cells showing the typical morphology (shrunken cells with condensed chromatin) as well as the cross-linked apoptotic bodies scored at light microscopy (Laborlux D; Leitz, Wetzlar, Germany) over 1000 total cells (including the apoptotic ones). In order to avoid subjective bias, the counts were carried out by different workers and the results pooled.

Establishment and characterization of L929 cell lines overexpressing tTG

In order to study the possible consequences of the accumulation of an inactive tTG in dying cells, an *in vitro* model system was established. L929 cells were co-transfected with the neomycin resistance gene (Neo) and an expression vector containing the human tTG cDNA, both driven by an SV40 early gene promoter. As previously reported in other cell lines (Melino *et al*, 1994; Gentile *et al*, 1992), the relative frequency of stably transfected tTG clones obtained was quite low and a high rate of spontaneous apoptosis was observed in the transfected cells. Two established clones were used (TG7 and TG8) for further studies along with a clone containing only Neo. The selected clones expressed human tTG mRNA as determined by *in situ* hybridization (Figure 5) and had high level of the enzyme protein as shown by the strongly positive immunohistochemical reaction with an antibody raised against human tTG (Figure 5). Enzyme activity measurements confirmed that wild type L929 cells and Neo cells have low enzyme activity (Figure 6A). The observed low levels of tTG activity seem to be responsible for the formation of low levels of ε-(γ-glutamyl)lysine cross-links detected in cellular protein (Table 2) as well as of the small number of detergent-resistant apoptotic bodies (Figure 6B). The two clones overexpressing tTG had about $5-6$ times greater tTG activity (Figure 6A). Similarly, the levels of cross-links in the protein fraction of these cells and the number of detergent resistant apoptotic bodies in the culture were much higher (Table 2 and Figure 6B).

Effect of TNFα on death, tTG activity and release of L929-derived cell lines

L929 cells are very sensitive to the cytotoxic action of TNFα, which in these cells results in massive cell death with the characteristic morphological and biochemical features of necrosis (Schulze-Osthoff *et al*, 1994). In our studies, TNFα treatment resulted in a slight increase of transglutaminase activity measured 24 h after the addition of TNFα (Figure 6A). However, the number of detergent resistant apoptotic bodies was higher in the TG-transfected clones (Figure 6B), suggesting that when the intracellular concentration of tTG

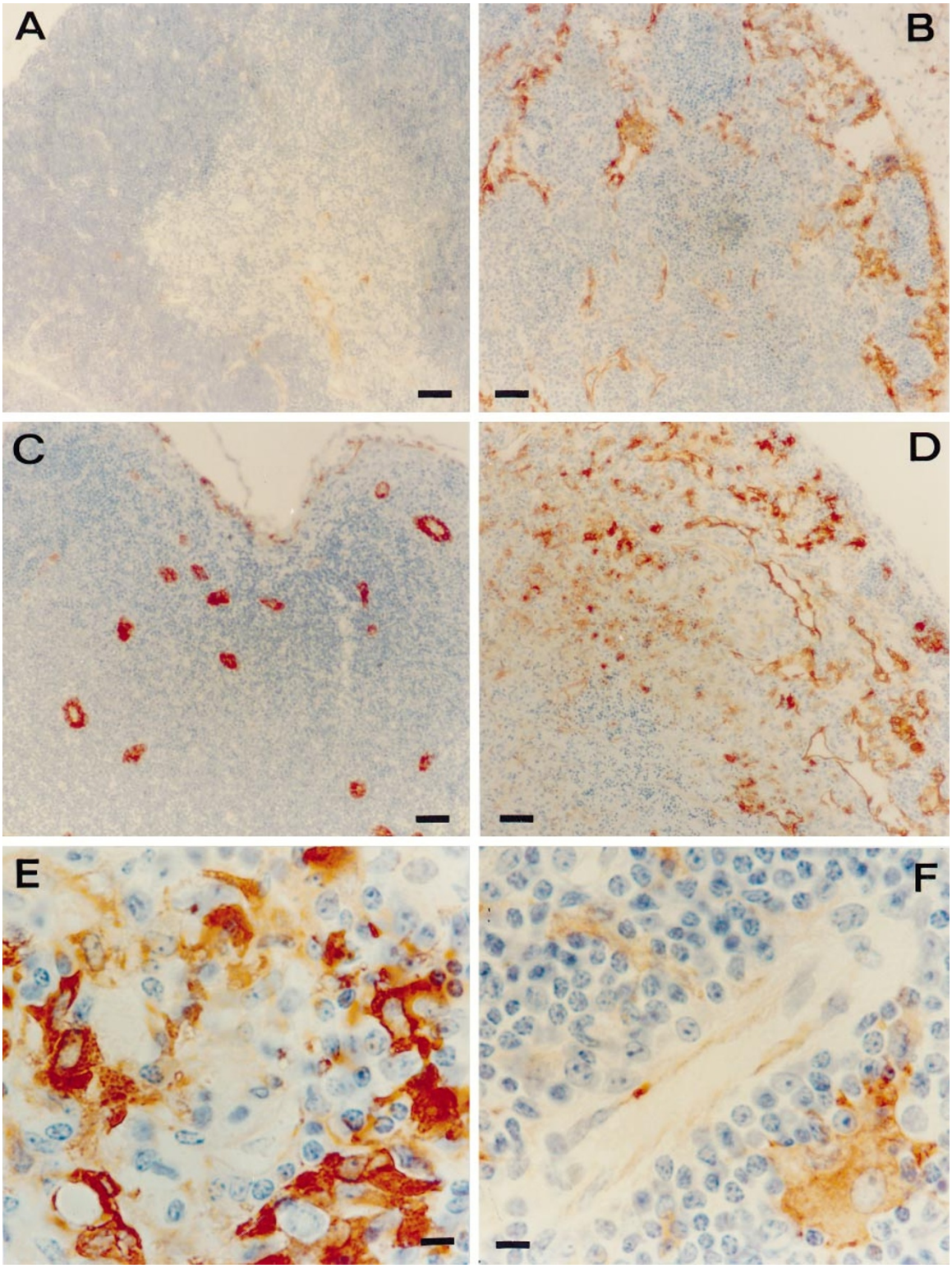

Figure 4 Immunohistochemical localization of tTG protein in thymuses (**A**–**B**) and lymph nodes (**C**–**F**) from 7 (**A**, **C**) and 30 (**B**,**D**–**F**) week-old MRL*lpr/lpr*-mice. Note the large increase in the tTG-specific staining observed in the cortex of the thymus of an old mouse (**B**) when compared with that of a young MRL*lpr/lpr* one (**A**). In the latter case only endothelial cells are stained by the tTG antibody (arrow heads). A dramatic increase in tTG staining is present in the stromal cells (arrows) of the lymph node of an old mouse (**D**) when compared with a young MRL*lpr/lpr* one (**C**). Panel **D** and **E** show a greater magnification of the lymph node from 30 week-old MRL*lpr/lpr* mice. Note the positivity of many polymorphic follicular dendritic-like cells (**E**–**F**). Bars: (**A**–**D**) = 80 μm; (**D**–**F**) = 10 μm.

is sufficient, cross-linked apoptotic bodies are formed even when cells die by necrosis. The high concentration of $\varepsilon(\gamma$-glutamyl)lysine cross-links in the TNFα treated tTG clones supports this notion (Table 2).

A key feature distinguishing necrosis and apoptosis is the leakage of intracellular macromolecules in the former and the retaining of these molecules in the apoptotic bodies in the latter (Fesus *et al*, 1991, Piacentini *et al*, 1994). L929

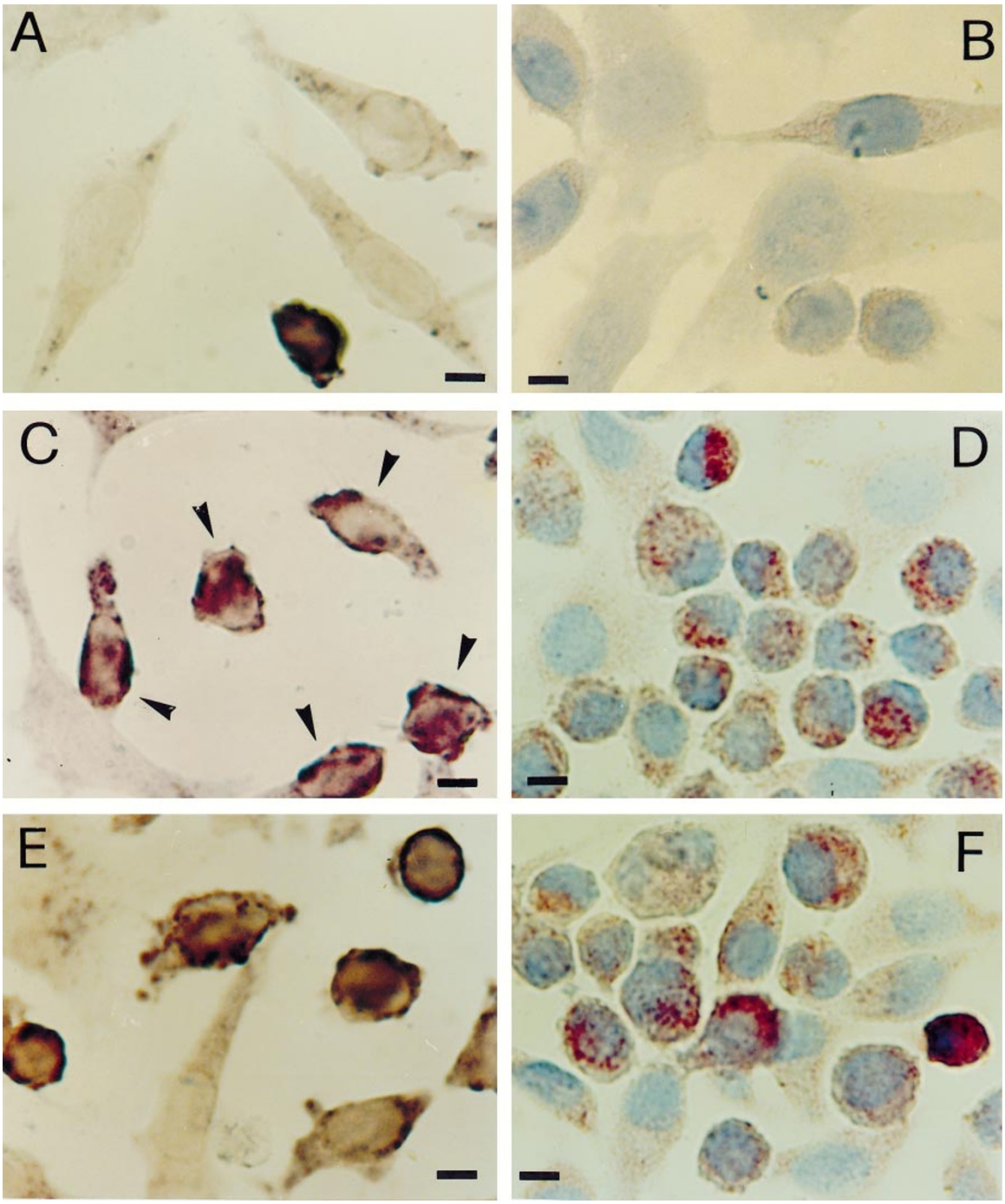

Figure 5 tTG expression in L929 transfected cells. L929 cells were transfected with pSV2Neo plasmid only (**A**–**B**) or cotransfected with pSV2Neo and pSG5-TGase constructs (**C**–**D**–**F**) and tTG expression was analyzed by *in situ* hybridization (**A**–**C**–**E**) and immunohistochemistry (**B**–**D**–**F**). Hybridization was carried out using a digoxygenin-labelled probe (human tTG cDNA), as described in Materials and Methods and immunocytochemically visualized by anti-digoxygenin Fab fragment labelled with alkaline phosphatase and colorimetric reaction. Note the absence of staining in viable substrate-adherent cells transfected with pSV2Neo plasmid only (**A**-arrows), and the intense positivity of cells cotransfected with the pSG5-TGase construct (**C**,**E**). The clone 7 (**C**–**D**) shows the presence of a high level of strongly positive apoptotic bodies (**C**, arrow heads), while the clone 8 (**E**–**F**) shows a lower level of tTGmRNA expression with respect to the clone 7, but always higher than the controls (compare **A** and **E**). Interestingly, the staining increased in parallel with the shrinkage typical of cells dying by apoptosis (**C**) Bars = 7 μm.

cells and their derived Neo clone lose a substantial portion of both their cytoplasmic and nuclear constituents during TNFα-induced necrotic-lytic death as detected by measuring the release of LDH and DNA into the extracellular space (Figure 7A and B, respectively). Despite the observed increased apoptotic rate (Table 2 and Figure 6B), the leakage of macromolecules was largely reduced in the tTG-transfected clones (Figure 7A and B, respectively).

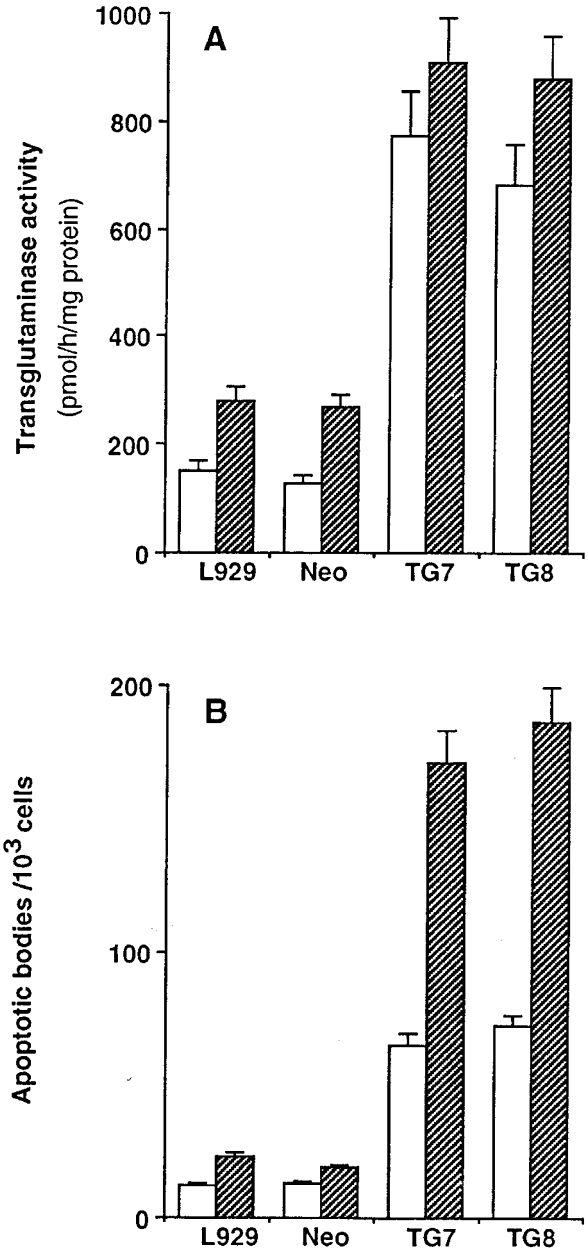

Figure 6 Effect of ectopic expression of tTG on apoptosis rate in L929 cells. Cells cultured as described in the Materials and Methods section were leave untreated (□) or treated with 250 IU/ml of TNFα (▨) for 24 h and tTG activity and the aoptotic index were measured. (**A**) *In vitro* tTG activity was measured as pmoles of [^{3}H]putrescine incorporated into protein/hour/mg protein. (**B**) tTG-dependent formation of cross-linked apoptotic envelopes was evaluated at the light microscope as described in Materials and Methods. Data are the mean of triplicate determinations with a S.E.M. in three different experiments.

However, when the tTG-transfected cells (Figure 7A and B) were treated with TNFα in the presence of a mechanism-based inhibitor of TG activity (which inhibits the intracellular formation of protein bound $\varepsilon(\gamma$-glutamyl)lysine cross-links; Table 1), the release of DNA and LDH from cells was again notably high (Killackey *et al*, 1989; Burton, 1956).

Detection of LDH in the blood of autoimmune MRL *lpr/lpr* mice

In order to verify as to whether these *in vitro* findings reflected of a real *in vivo* pathological condition we analyzed the LDH levels during the development of autoimmunity in MRL *lpr/lpr* mice. Table 3 shows that mice bearing the mutation in the CD95 receptor indeed have much higher LDH levels in the plasma when compared to control mice. It is important to note that this increased LDH release was detected in young mice (7 weeks old) which already displayed a reduced tTG specific activity (Table 1), but do not yet present circulating tTG autoantibodies, lymphadenopathy, glomerulonephritis, arteritis and arthritis occurring with the development of the autoimmune syndrome.

Discussion

The biological importance of apoptosis and the role of its perturbance in several human diseases (Thompson, 1995) has recently elicited a great deal of interest in defining the biochemical mechanisms which control the initiation of this complex cellular phenomenon (Fesus *et al*, 1991; Piacentini *et al*, 1994). By contrast, much less attention has been focused on the biochemical events characterizing the late stages of apoptosis, even though they have fundamental importance in achieving a controlled and 'social' execution of the cell (Kerr, 1995). In this study we show that the presence of a functional tTG activity is necessary for a correct program of apoptosis. A peculiar feature of cells dying by apoptosis is the maintained cellular integrity before phagocytosis and the subsequent degradation by adjacent cells. The biochemical processes underlying the structural changes occurring in the cytoplasm of apoptotic cells are still largely unknown. We have demonstrated here that tTG-catalyzed intracellular protein cross-linking plays an important role in increasing the stability of apoptotic bodies and the consequent reduction of leakage of their contents. In fact, overexpression of tTG is able to reduce

Table 2 $\varepsilon(\gamma$-glutamyl)lysine concentration in the protein fraction of wild type and tTG-transfected L929 cells following treatment with TNFα and/or a tTG inhibitor

Treatments	None	tTG inhibitor	TNFα	TNFα+tTG inhibitor
L929	305±41	228±30	383±39	262±27
Neo	275±41	230±27	363±34	268±22
TG 7	950±82	439±54	3805±223	1486±297
TG 8	870±69	474±66	4165±318	1379±287

Cells cultured as described in the Materials and Methods section were treated with 250 IU/ml of TNFα and/or the transglutaminase inhibitor (200 μM; Syntex RS 71666-007) for 24 h. $\varepsilon(\gamma$-glutamyl)lysine concentration was detected after exhaustive proteolytic digestion of the protein fraction as previously reported (Fesus *et al*., 1991; Harsfalvi *et al*., 1992). Data are expressed as pmol/mg protein and are the mean with a S.E.M. of four different experiments.

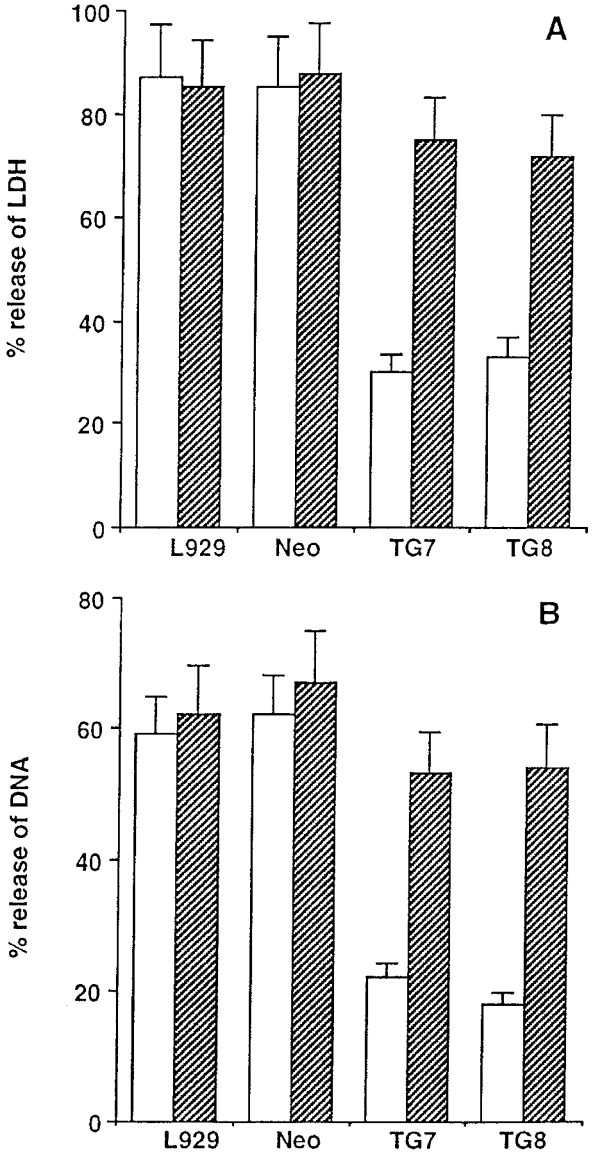

Figure 7 Effect of TNFα on the LDH and DNA release in tTG-transfected L929 cells. Cells cultured as described in the Materials and Methods section were treated with 250 IU/ml of TNFα alone (□) or in the presence of (▨) transglutaminase inhibitor (200 μM; Syntex RS 71666-007) for 24 h and LDH (**A**) and DNA (**B**) release quantified as previously described. Data are the mean of triplicate determinations with a S.E.M. in four different experiments.

Table 3 Effect of aging on LDH levels in the blood of MRL+/+ and MRL*lpr/lpr* mice

Age	LDH*	
(weeks)	MRL+/+	MRL*lpr/lpr*
7	1.3±0.4	10.0±0.5°
30	0.8±0.3	7.1±0.3°

(*) LDH enzymatic activity is expressed as mU/mg protein and was measured as described by Howell *et al* (1979). Data are the mean of triplicate determinations with a S.E.M. in four different animals. (°) Statistically significant ($P<0.01$) with respect to the MRL+/+ mice.

the release of macromolecules (DNA and LDH) characterizing TNFα-induced necrotic death of L929 cell line. This protective effect is abolished when the crosslinking activity of the enzyme is blocked by a specific inhibitor. The biological relevance of protein cross-linking is particularly evident in the programmed death of keratinocytes in squamous epithelia. In these cells various substrate proteins (involucrin, loricrin and others) are cross-linked by specific transglutaminases to form an intracellular protein 'envelope', beneath the plasma membrane of the dying corneocyte: the structure which isolates and protects our organism from external insults (Polakowska and Haake, 1994; Steinert, 1995).

MRL*lpr/lpr* mice have abnormally high levels of TNFα (Nagata and Golstein, 1995, Izui *et al*, 1984) which, in the presence of low levels or inactive tTG detected in these animals, might lead to lytic death similar to necrosis in some cells. Hence, at both the cellular and the molecular levels, the autoimmune pathology observed in MRL*lpr/lpr* mice seems to reproduce various aspects of the necrotic death described in L929 cells *in vitro* (Schulze-Osthoff *et al*, 1994), suggesting that release of macromolecules from cells lacking tTG may indeed take place in these autoimmune mice. We have shown here, that the accumulation of autoantibodies against tTG, 70 kDa HSP and dsDNA takes place only in old MRL*lpr/lpr* mice, while increased LDH levels are already detectable in the blood of young MRL*lpr/lpr* mice. These findings suggest that the continuous presence of circulating intracellular autoantigens, very likely due to a necrotic *versus* an apoptotic form of death, might contribute to the development of autoantibodies observed in old MRL*lpr/lpr* mice. In fact, a massive autoimmune response, such as that of MRL*lpr/lpr* mice, requires a long lasting defective stimulation of the immune system (Theofilopoulos, 1995); which, for apoptosis impairment, may be partially explained by the CD95 mutation and the presence of an inactive tTG. However, the early deregulation of other elements of the immune system is very likely required to fully explain the molecular basis of a complex disease such systemic autoimmunity. It is interesting to note that the presence of increased levels of plasmatic LDH has also reported in a human autoimmune syndrome (Gemma *et al*, 1992).

In addition to tTG and dsDNA, the presence of autoantibody to lipocortin-1 (LC1) has also recently been demonstrated in the autoimmune-prone MRL mice (Ikai *et al*, 1992). LC1 is a well characterized tTG substrate, which is induced during apoptosis (McKanna, 1995; Ando *et al*, 1989). LC1 specifically binds to Ca^{2+} and phosphatidylserine and displays marked anti-inflammatory and immunosuppressive features (McKanna, 1995; Ando *et al*, 1989). The affinity for Ca^{2+}- and phosphatidylserine and consequently the biological activity of LC1 are regulated by its tTG-dependent polymerization (Ando *et al*, 1989). Hence, the lack of a functional tTG, observed in the autoimmune MRL*lpr/lpr* mice, may lead to the inhibition of LC1 biological activity with marked effects on inflammation and autoimmune responses. In addition, the phagocytosis of apoptotic cells involves the recognition of phosphatidylserine specifically exposed on the surface of dying cells (Savill *et al*, 1993). Since, polymerized-LC1 specifically binds to phosphatidylserine, an important role for this tTG substrate

in the clearance of apoptotic cells may be also envisaged. Future studies should rule out whether the absence of the tTG-dependent polymerization of LC1 might have a role in the abnormal accumulation of 'preapoptotic' cells in the lymphoid organs of autoimmune MRL*lpr/lpr* mice (Van Houten and Budd, 1992).

The abnormal accumulation of tTG in viable accessory cells of lymphoid tissues suggests a generalized disregulation of apoptosis in the immune system of MRL*lpr/lpr* mice. An important role for the L3T4 T lymphocyte subset in the development of autoimmunity in *lpr* mice has been proposed (Santoro *et al*, 1988). This specific inducer/helper autoreactive T cell clone once activated by antigen presenting cells (APCs) expressing the appropriate Ia allele and antigen, autonomously kills its target APC (Ju *et al*, 1986). Thus, in the absence of a functional CD95 receptor in *lpr* mice this subset of T cells might deliver an incomplete death signal leading to partial induction of the apoptotic program which involves the expression of an inactive tTG in stromal cells.

Two possible explanations for the presence of catalytically-inactive tTG can be hypothesized. Nakaoka *et al*. (1994) have demonstrated that tTG is the 74 kDa subunit ($G_{\alpha h}$) associated with the 50 kDa β subunit ($G_{\beta h}$) of the G_h protein (Nakaoka *et al*, 1994). Thus, the $G_{\alpha h}$ is a multifunctional protein which, by binding GTP in a $G_{\alpha hGTP}$ complex, modulates receptor-stimulated phospholypase-C (PLC) activation and inhibits its transglutaminase activity which is needed to prevent the release of macromolecules from cells dying by apoptosis. tTG-catalyzed reactions result in post-translational modification of proteins by establishing $\varepsilon(\gamma$-glutamyl)lysine cross-linkings and/or covalent incorporation of di- and polyamines into proteins (Folk, 1980; Piacentini *et al*, 1994). Diamines and polyamines may also participate in cross-linking reaction through the formation of N,N-bis(γ-glutamyl)polyamine cross-bridges (Folk, 1980; Piacentini *et al*, 1994). We demonstrated that high intracellular levels of putrescine, spermidine and spermine might impair the stabilizing cross-linking activity of tTG by favouring the formation of mono(γ-glutamyl)polyamine derivatives (Piacentini *et al*, 1990). Consistent with this hypothesis increased levels of di- and polyamines during the development of autoimmunity in MRL*lpr/lpr* mice have recently been demonstrated (Hsu *et al*, 1994).

In conclusion, these studies indicate that deregulation of the cell death program plays a critical role in the development of autoimmunity. In addition to the defect in the CD95 system, we have shown that the impairment of other apoptosis-related gene products, such as tTG, may be one of the component in the complex mechanism leading to the production of self-antigens and to autoimmune responses.

Materials and Methods

Chemicals

{1,4 (n)-^{3}H} putrescine dihydrochloride (26.3 Ci/mmol) was obtained from Amersham, Bucks, UK). Optifluor was obtained from Packard (Zurich, Switzerland). N,N'-dimethylcasein, bovine serum albumin, putrescine hydrochloride and dexamethasone were from Fluka (Buchs, Switzerland). Strepavidin-Biotin immunoperoxidase staining systems were from Biogenex (USA). Tumor Necrosis Factor-α (TNFα) and cell culture reagents were from Sigma (St. Louis, USA). The mechanism-based transglutaminase inhibitor (RS 71666-007) was obtained from Syntex Research, Canada (Castelhano *et al*, 1998; Killackey *et al*, 1989). Other chemicals were of reagent grade and used without further purification. DNA release was measured as described previously (Burton, 1956). Lactate dehydrogenase activity was determined by an UV kinetic method using a commercial kit supplied by Sigma (St. Louis, USA).

Cell cultures and transfection

L929 murine cells were grown in RPM-I 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf-serum, 2 mM L-glutamine, 2 g bicarbonate per liter, in a humidified atmosphere with 5% (vol/vol) CO_2 at 37°C. L929 cells were transfected with pSV2Neo or co-transfected with pSG5-TGsense and PSV2Neo constructs as described previously (Melino *et al*, 1994). For selection, cells were cultured in the presence of 0.8 mg/ml of G418 and cell clones were obtained from individual G418-resistant colonies. Cells cultured were treated with 250 IU/ml TNFα and/or with 200 μM transglutaminase inhibitor (RS 71666-007) for 24 h.

Mice

The following mouse strains were used: MRL/Mp-*lpr/lpr* (MRL*lpr/lpr*), MRL/Mp-+/+ (MRL+/+) and CBA at 7, 10, 20, 30, 50 weeks of age. All mice were obtained from Cave-Tech Breeding Unit and were maintained as continuously breeding colonies in the local animal facility. Sera were prepared on Microtainer (Becton Dickinson, Mountain View, CA) from blood taken from animals under anesthesia by cutting of subclavian vein and brachial artery, aliquoted and stored at -20°C until use.

Enzyme linked immunosorbent assay (ELISA)

Microtiter plates (Becton Dickinson) were coated overnight at room temperature with purified human red blood cell tTG (Fesus and Arato, 1986) or with the peptide corresponding to the residues 235–249 of Heat Shock Protein (70-HSP-01): amino acid sequence NRLVNHFVEEFKRKH, purity more than 90% by HPLC analysis. For anti-dsDNA microplates were coated with 100 μl/well of poly L-Lys (Sigma, USA) at the concentration of 5 μg/ml for 1 h at room temperature; after five washes in plates, 100 μl of fetal bovine thymus dsDNA (Sigma, USA) were diluted in phosphate buffer saline (PBS) at a concentration of 1 μg/well in 100 μl of 0.05 M sodium carbonate buffer, pH 9.5. Coated plates were washed five times with 0.9% NaCl containing 0.05% Tween 20. Mouse sera, diluted 1 : 100 with PBS containing 0.5% bovine serum albumin, 0.05% Tween 20, were added to coated wells and incubated at 37°C for 60 min. After washing a peroxidase-labeled goat anti-mouse Ig (BIO-RAD, USA, recognizing heavy and light chain of IgG), diluted 1 : 2000 was added. All incubations were performed at 37°C for 60 min, followed by five washes. After the last incubation, horseradish peroxidase substrate (1,2-phenylenendiamine, DAKO, Denmark) in citrate-phosphate buffer, pH 5, and 0.015% H_2O_2 was added and allowed to react for 30 min at 37°C; reaction was stopped by adding 50 μl 1M HCl, and the light absorbance was determined at 490 nm in an ELISA reader (BIO-RAD, USA). Results were expressed as the mean $\pm$ standard error of at least three determinations for each experimental group.

'Tissue' transglutaminase assay

Thymuses and lymph nodes from CBA mice (7 and 50 weeks old), MRL+/+ and MRL*lpr/lpr* mice (7 and 30 weeks old) were washed extensively with PBS (without Ca^{2+} and Mg^{2+}) and homogenized in 0.1 M Tris-HCl, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, 1 mM PMSF. L929 cells after different treatment were mechanically removed from flasks, washed with PBS and sonicated at 4°C for 20 s. tTG activity was measured by detecting the incorporation of [^{3}H]putrescine into N,N'-dimethylcasein as previously reported (Piacentini *et al*, 1986). Protein concentration was determined using bovine serum albumin as standard.

In situ hybridization

Fixation of tissue culture cells L929 cells transfected with pSV2Neo plasmid or cotransfected with pSV2Neo and pSG5TGSense were grown for 3 days on coverslips, removed from the growth media and fixed in a freshly prepared 4% paraformaldeyde.

tTG DNA probe A 3.4 Kb fragment containing mouse tissue transglutaminase was used as cDNA probe (pmT700pU probe), labelled by the incorporation of digoxygenin-labelled deoxyuridine triphosphate (Dig-dUTP) (Boehringer Mannheim, Germany) by random prime DNA labelling. To check the activity of the probe, it was diluted in distilled water (5 ng–0.1 pg/50 μl) spotted onto nitrocellulose filters and visualized immunoenzymatically as described below.

Hybridization and detection of hybridized tTG probe *In situ* nucleic acid hybridization was performed according to the method described by Brigati *et al* (1983) with minor variations.

Samples were overlaid with 10 μl of prehybridization buffer (50% Formammide, 5 × Denhardt's, 100 μg/ml carrier salmon sperm DNA in 6 × SSC) for 15 min at 42°C. Following prehybridization, the labelled probe (2 μg/ml) was denaturated by heating at 85°C for 6 min and then added in hybridization mixture and allowed to hybridize for 12 h at 45°C. After hybridization, the coverslips were carefully removed and samples were washed at 37°C in 2 × SSC, 2 × SSC, 0.1 × SSC for 15 min each.

Subsequently samples were processed for detection using a polyclonal antidigoxigenin Fab fragment from sheep labelled with alkaline phosphatase (Boehringer). The colorimetric reaction was developed with nitroblue tetrazolium salt as alkaline phosphatase substrate. The development of a dark purple coloured precipitate at the enzyme site was monitored by microscopic examination.

Western blot analysis

Determination of tTG protein was carried out on lymph nodes and thymuses from CBA mice (7 and 50 week-old), MRL+/+ and MRL*lpr/lpr* mice (7 and 30 week-old). Extracts (100 μg of total protein) were run on 10.5% SDS–PAGE (Melino *et al*, 1994). The gel was electroblotted onto nitrocellulose paper at 40 mA overnight. tTG positive bands were revealed by utilizing an affinity purified IgG raised in rabbits against human tTG as a primary antibody and, horseradish peroxidase conjugated goat anti-rabbit IgG (BIO-RAD, USA) as a secondary antibody. The reaction was developed by the chemoluminescence ECL detection system (Amersham, UK).

Immunohistochemistry

Immunohistochemical staining was performed using an affinity purified monospecific IgG raised in rabbits against human red blood cell soluble tTG (1 : 100) as a primary antibody (Fesus and Arato, 1986). Incubations with the primary antibody were carried out in a wet chamber overnight at 4°C. A biotinylated goat anti-rabbit IgG was used as secondary antibody, followed by a preformed strept-avidin-horseradish peroxidase complex (BioGenex, USA). The reaction was developed using aminoethylcarbazole (AEC), (CRL, USA) as chromogen substrate and 0.01% H_2O_2. Slides were counterstained in Mayer's haematoxylin. Endogenous peroxidase activity was blocked by methanol-H_2O_2.

Evaluation of the apoptotic index

To estimate the apoptotic index in lymph node from 10, 20 and 30 weeks old MRL*lpr/lpr* mice, cells freshly isolated were smeared on slides and fixed with 2.5% paraformaldehyde and immunostained by anti-tTG antibody. The percentage of apoptotic cells was evaluated by counting the tTG positive cells showing the typical morphology (shrunken cells with condensed chromatin) as well as the cross-linked apoptotic bodies scored at light microscopy (Fesus *et al*, 1989) Laborlux D; Leitz, Wetzlar, Germany) over 1000 total cells (including the apoptotic ones). In order to avoid subjective bias, the counts were carried out by different workers and the results pooled.

$\varepsilon(\gamma$-glutamyl)lysine measurement

$\varepsilon(\gamma$-glutamyl)lysine measurement was carried out according to a previously published method (Fesus *et al*, 1991; Harsalvi *et al*, 1992). A preliminary purification of amino acids and peptides was achieved by eluting aliquots of the plasma samples on ion-exchange chromatography and on silica column. Separation of amino acids and peptide derivatives was carried out by HPLC on a mBundapack C18 column upon derivativization with phenylisothiocyanate (PITC); ^{3}H-labelled $\varepsilon(\gamma$-glutamyl)lysine was used as an internal standard throughout the procedure. The quantification of $\varepsilon(\gamma$-glutamyl)lysine in the plasma was based on peak area as compared to those obtained from a standard amount of the $\varepsilon(\gamma$-glutamyl)lysine (Serva; Germany) and on the recovery determined by isotope dilution.

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