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## Granule associated DNase in T4 and T8 lymphocytes from patients with autoimmune diseases

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### Abstract

The presence of a DNase activity associated with secretion granules was detected in T4 and T8 lymphocytes from patients with autoimmune diseases. This activity was much higher in primary biliary cirrhosis (PBC) than in Graves' disease (GD) and multiple sclerosis (MS) or in healthy subjects. This granule associated DNase activity was Ca<sup>2+</sup>-dependent, inhibited by Zn<sup>2+</sup>, and higher at low pH; its molecular weight corresponded to 66 kDa; it was more active with double-strand than single-strand DNA. Judging from its properties this enzyme differed from the three types of endonucleases described as involved in DNA fragmentation (DNase I, DNase II and NUC18). Flow cytometry analysis of T lymphocytes showed that DNase activity associated with CD4<sup>+</sup> lymphocyte granules correlated with the ratio CD4<sup>+</sup>CD45RO<sup>+</sup>/CD4<sup>+</sup>CD45RA<sup>+</sup> (memory and cytotoxic cells/naive cells, inducers of suppression). In contrast, T8 lymphocyte DNase activity correlated with the proportion of CD4<sup>+</sup> lymphocytes with CD4<sup>+</sup>CD45RA<sup>-</sup> phenotype (helpers and inducers of cytotoxicity). The possible role of this DNase activity in the mechanisms of lysis or apoptosis mediated by CTL is discussed. We suggest that this DNase activity could be implicated in some of the alterations of the autoimmune response depending on cytotoxic T lymphocytes or T cell inducers of apoptosis. © 1998 Elsevier Science B.V.

**Keywords:** Granule associated DNase activity; Cytotoxic T lymphocytes; Graves' disease; Primary biliary cirrhosis; Multiple sclerosis

### 1. Introduction

Organ-specific autoimmune diseases are characterized by abnormal immune responses; the immune system attacks autologous tissues leading to profound

alterations. In some of these diseases, such as primary biliary cirrhosis (PBC) or multiple sclerosis (MS), tissue infiltration by both CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes, some of them with cytotoxic activity (cytotoxic T lymphocytes; CTL), at the organs affected are a common occurrence, and for that reason considered to be T-cell-mediated autoimmune diseases [1–4]. Although autoantibodies against TSH receptor are crucial in the stimulation of the thyroid gland in Graves' disease (GD) [5], intrathyroidal lymphocytes may also participate in the induction of thyroid dysfunction [6,7]. Some authors have sug-

Abbreviations: PBC, primary biliary cirrhosis; GD, Graves' disease; MS, multiple sclerosis; HD, healthy donor; CTL, cytolytic T lymphocytes; PBMC, peripheral blood mononuclear cells; F.I., fragmentation index; DTT, dithiothreitol

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gested that an imbalance among different T cell subsets may be an underlying cause leading to autoimmunity [8–11].

Lymphocyte-mediated cytolysis is a multistage process possibly involved in the destruction of target cells in a major histocompatibility complex class I and class II restricted attack with the participation of auxiliary adhesion molecules [12–14]. Two possible cytotoxic mechanisms, not mutually exclusive, probably associated with different types of CTL, or with the same effector cell at different stages of differentiation, have been proposed [13,14]: in the first mechanism, lytic molecules, prepackaged in cytoplasmic granules are secreted to form lytic pores on the target cell membrane, whereas in the second mechanism, interactions between specific cell surface receptors trigger apoptosis. Cooperation between secreted granzymes and perforin brings about CTL-induced lysis, including DNA fragmentation; in addition, HLA-II restricted perforin-negative CD4<sup>+</sup> CTLs could also be responsible for this lysis [14]. A 15 kDa cytoplasmic granule associated protein, the T cell-restricted intracellular antigen (TIA-1), mainly associated with a subpopulation of CD8<sup>+</sup> T lymphocytes and scarcely present, or not present at all, in CD4<sup>+</sup> resting T lymphocytes has been described [15]. However, it has been recently described that CD4<sup>+</sup> CTLs simultaneously induce apoptosis of target cell nuclei and membrane damage; CD4<sup>+</sup> clones synthesize perforin and granzyme B and express the granule associated protein TIA-1 [16], indicating that two distinct mechanisms may contribute to cytolysis by CD4<sup>+</sup> clones: a Ca<sup>2+</sup>-dependent mechanism associated with cytotoxic granules and a Ca<sup>2+</sup>-independent mechanism. TIA-1, expressed preferentially on cells with cytolytic potential, has been found to induce DNA fragmentation in digitonin permeabilized thymocytes, suggesting that this antigen might be the granule component responsible for triggering apoptosis in target cells [17]. It has also been reported [18] that in inflammatory muscle diseases of autoimmune origin a chronic lymphocyte infiltration is always present around the muscles damaged. Cytotoxic cells can induce target cell death by the two mechanisms mentioned above. Granzyme B, perforin and TIA-1 protein were expressed simultaneously on muscle samples from patients with polymyositis, suggesting that cytotoxic cells can cause muscle damage and muscle

necrosis and/or apoptosis by releasing proteins responsible for the lysis of target cells [18].

We have already reported the expression of DNase activity associated with cytoplasmic granules on activated CD4<sup>+</sup> lymphocytes after the incubation of PBMC from healthy subjects in the presence of immunomodulating peptides [19]. A number of endonucleases have been described in a variety of cells, some of them related to DNA fragmentation in apoptosis [20–22]. The induction of this activity in human activated CD4<sup>+</sup> lymphocytes [19] and the participation of T cells in autoimmune diseases [1–4] prompted us to explore the possible presence of granule associated DNases in T lymphocytes obtained from patients with autoimmune diseases. We report now the existence of a DNase activity associated with cytoplasmic granules in CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from patients with this type of diseases. This acidic DNase of 66 kDa, activated by Ca<sup>2+</sup> and inhibited by Zn<sup>2+</sup>, required double-strand DNA as substrate. The correlation of this activity with different T cell subsets suggests that this enzyme activity could be involved in the immunological alterations observed in autoimmune diseases mediated by CTL activity.

## 2. Materials and methods

### 2.1. Subjects

All patients with clinically defined PBC, MS or GD included in this report are part of a follow-up study being carried out at the University Clinic of the University of Navarra (Spain). We studied 10 patients with PBC (female/male: 8/2; age  $\pm$  standard deviation: 53  $\pm$  13), 10 patients with MS (7/3; 43  $\pm$  9), 10 with GD (9/1; 47  $\pm$  9), and 15 healthy donors (9/6; 39  $\pm$  15). Patients were randomly selected with the purpose of finding individuals in different stages of each of these diseases. 7 PBC patients were in stage I and 3 in stage IV. 6 of the MS patients were in an active phase, and in the remaining 4 the disease was stabilized and had no exacerbation for at least 3 months before the beginning of the study. All of the GD patients studied had circulating thyroid antibodies and high levels of T4 at the moment of blood sampling.

## 2.2. Isolation of lymphocytes

PBMC were isolated from heparinized venous blood by Ficoll–Hypaque density gradient. CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes were separated from  $3 \times 10^7$  PBMC after incubating the whole PBMC population with magnetizable polystyrene beads coated with a primary mononuclear antibody for CD4 or CD8 membrane antigen (Dynal, Oslo, Norway). CD4<sup>+</sup> lymphocytes were obtained from 15 healthy donors (HD), 7 PBC patients, 5 MS patients, and 5 GD patients; CD8<sup>+</sup> lymphocytes from 15 HD, 9 PBC patients, 5 Ms patients and 8 GD patients.

## 2.3. Extraction of granule proteins

The isolation of secretion granules was carried out using a Percoll density-gradient after disrupting the cells with a Parr nitrogen bomb. Granule proteins were extracted following the procedure of Podack et al. [23].

## 2.4. Detection of DNase activity

Detection of DNase activity in protein extracts was performed by the ability to degrade genomic DNA. The extracted granule proteins corresponding to  $3 \times 10^7$  PBMC were incubated with 10 µg of protein free DNA at 37°C for 24 h in 50 µl of a 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 10 mM Tris–HCl autoclaved buffer, pH 7.5. Some incubations were performed without the addition of DTT. When testing at pH values other than 7.5, the following buffers were used: sodium acetate (pH 5), MES (pH 6), MOPS (pH 7) or HEPES (pH 8) all of them at 10 mM. An aliquot of 20 µl of the resulting mixture was applied to a 1% agarose gel, and subjected to electrophoresis at a voltage of 50 V in 1 mM EDTA, 40 mM Tris–HCl buffer, pH 7.5, containing 0.5 µg of ethidium bromide per ml. Genomic DNA was obtained as described in the literature [24]. To quantify and compare the results obtained in different experiments, we carried out a simple image analysis with the appropriate software (Image Master, Pharmacia). Electrophoregrams on gels were photographed and images scanned and inverted. Each lane was divided into three sectors, using as reference a molecular weight marker (*Hind*III-digested 1 phage

DNA, Promega) loaded in parallel to the samples of each gel. Sector A corresponded to the 23 Kb first band of the marker and contained the undigested DNA; sector B extended from the lower limit of sector A to the top of the 2.3 Kb band of the marker, and contained DNA partially degraded in fragments larger than 2.3 Kb; sector C extended from the lower limit of sector B to the end of the lane and contained DNA degraded in fragments smaller than 2.3 Kb. The programme integrates the absorbance of each sector and compares it with the total absorbance of each lane taken as 100%. This allows us to compare samples run on different gels, and calculate a “fragmentation index” (F.I. (%) =  $1/2 B (\%DNA > 2.3 \text{ Kb}) + C(\%DNA < 2.3 \text{ Kb})$ ). In each case, a digestion in DNase free medium was performed in parallel to calculate the spontaneous degradation which was subtracted from F.I.

## 2.5. DNase detection in DNA–SDS–PAGE

Protein electrophoresis was carried out following the technique of Laemmli and Favre [25]. Separating gels (12% acrylamide–bisacrylamide 37.5/1) were prepared with the addition of calf thymus DNA 10 µg/ml. Protein extracts were mixed with sample loading buffer (0.125 M Tris–HCl, pH 6.4, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue) and heated at 37°C for 20 min before being placed in the wells. Following electrophoresis, gels were washed three times in distilled water, once in 40 mM Tris–HCl, pH 7.6, 2 mM MgCl<sub>2</sub>, 1 mM EDTA and twice again in distilled water. Each wash was prolonged for 10–12 min in order to ensure the removal of SDS and β-mercaptoethanol. Incubation was continued in 10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 1 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, 1 mM DTT. Experiments were also carried out in the absence of DTT. After 4 h ethidium bromide (1 µg/ml) was added to the gel incubation buffer. After 30 min gels were examined under UV light and photographed. The positions of DNase activity were revealed as dark bands on a fluorescent background.

## 2.6. Analysis of lymphocyte subpopulations

Phenotypic characterization of T cells was carried out using an EPICS Profile-II flow cytometer (Coulter

Electronics, Hialeah, FL, USA). The monoclonal antibodies used in this work were supplied by Becton–Dickinson (Becton-Dickinson Immunocytometry Systems, Erembodegen, Belgium). Analysis of membrane determinant co-expression using double-colour combinations (Fluorescein and Phycoerythrin) was done following the manufacturer's instructions. To determine TIA-1 antigen in human lymphocytes murine macrophage IgG1 anti-TIA-1 was used (Coulter Electronics, Hialeah, FL, USA).  $5 \times 10^5$  lymphomononuclear cells were fixed with cold 4% paraformaldehyde for 20 min, then washed with PBS containing 0.1% sodium azide and suspended in 50  $\mu$ l of permeabilization buffer (PBS with 0.1% sodium azide, 1% heat-inactivated FCS and 0.1% saponin). Cells were incubated with anti-TIA-1 antibody at 4°C for 45 min, and washed with permeabilization buffer. Cells were then incubated again for 30 min in the presence of rat antibody against murine IgG1 conjugated with phycoerythrin. After washing them with permeabilization buffer, cells were suspended in PBS

and analyzed by flow cytometry. The expression of TIA-1 on T4 or T8 lymphocytes was analyzed using double color analysis.

### 2.7. Statistical analysis

The values obtained from cytometry were analyzed by U Mann-Whitney test to compare data between groups of subjects. Correlations were analyzed by the Spearman test. A *p* value less than 0.05 was considered to be significant.

## 3. Results

### 3.1. DNase activity in cytoplasmic granules of $CD4^+$ and $CD8^+$ T cells

Protein extracts from secretion granules corresponding to T4 and T8 lymphocytes isolated from

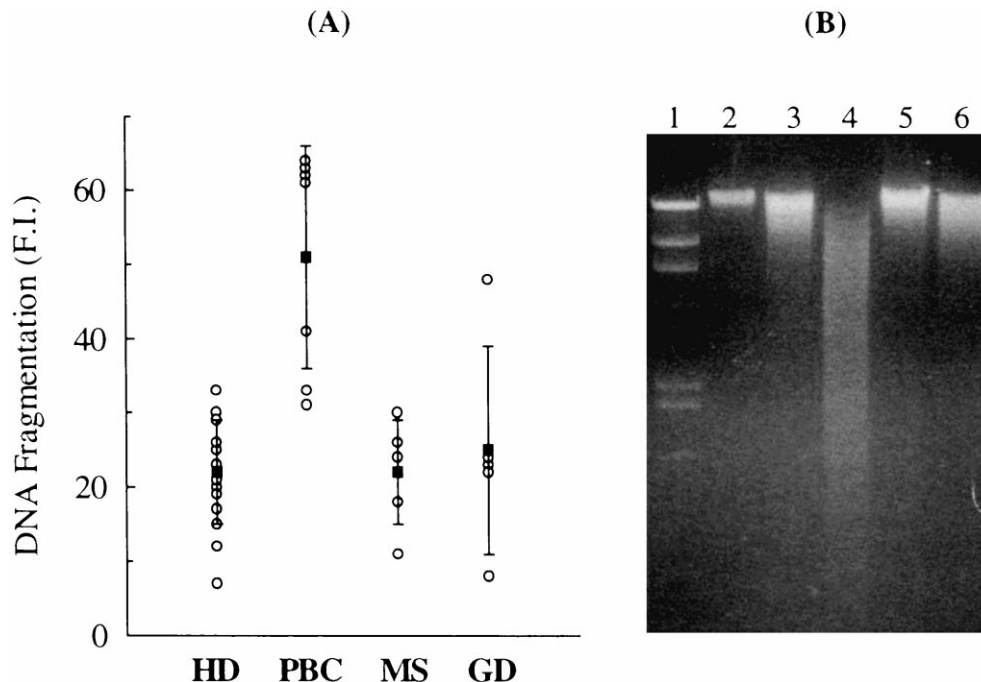


Fig. 1. DNase activity in granules of  $CD4^+$  cells from PBC, MS, GD and healthy donors. (A) DNase activity from  $CD4^+$  cells expressed as 'fragmentation index' (F.I.). All the individual values are represented together with the mean  $\pm$ S.D. for each group studied. (B) Electrophoregram reflecting DNase activity extracted from granules present in  $CD4^+$  cells. For each group a representative example is given. Lane 1, Standard DNA. Lane 2, DNA incubated with DNase-free medium. Lanes 3, 4, 5 and 6, DNA fragmentation induced by DNase present in  $CD4^+$  cells from a healthy donor, or from PBC, MS and GD patients, respectively.

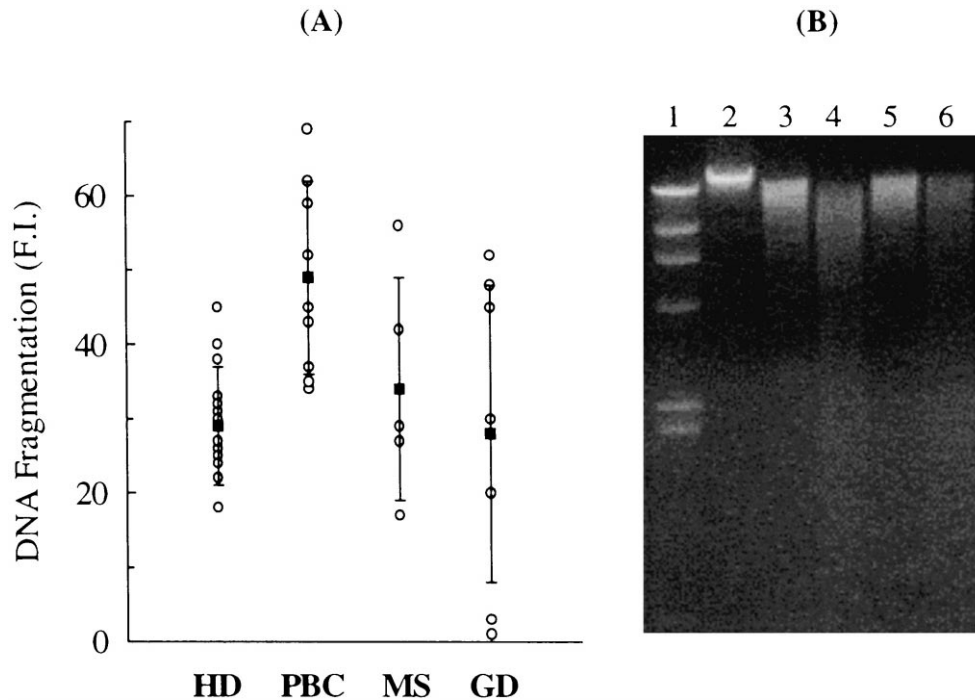


Fig. 2. DNase activity in granules of CD8<sup>+</sup> cells from PBC, MS, GD patients and healthy donors. (A) DNase activity from CD8<sup>+</sup> cells measured as “fragmentation index” (F.I.). All the individual values are represented together with the mean  $\pm$  S.D. for each group studied. (B) Electrophoregram reflecting DNase activity present in granules of CD8<sup>+</sup> cells. For each group a representative example is given. Lane 1, Standard DNA. Lane 2, DNA incubated with DNase-free medium. Lanes 3, 4, 5 and 6, DNA fragmentation induced by DNase present in CD8<sup>+</sup> cells from a healthy donor, or from PBC, MS and GD patients, respectively.

$3 \times 10^7$  fresh PBMC belonging to patients or healthy donors were incubated with protein-free genomic DNA for 24 h. Then DNase activity was determined analyzing DNA fragmentation by electrophoresis on agarose gel. A higher DNase activity, expressed as “fragmentation index” (F.I.), was found both in CD4<sup>+</sup> (Fig. 1) and CD8<sup>+</sup> cells (Fig. 2) from patients when compared to HD;  $p = 0.0431$  and  $p = 0.0459$ , respectively. The highest values were found in PBC patients. In some GD patients a high DNase activity was also found; although the average of all the values of DNase activity was not higher than in HD, individual variability was very wide, perhaps reflecting different immunological situations typical of this disease. In MS patients the F.I. values were similar to those found in HD.

### 3.2. Properties of the DNase enzyme

DNase activity, both in CD4<sup>+</sup> and CD8<sup>+</sup> cells, increased when Ca<sup>2+</sup> was present in the medium at

pH 7.5. Fig. 3 shows the fragmentation of DNA in a representative experiment using CD4<sup>+</sup> cells as source for the enzyme. This experiment was repeated seven times with CD4<sup>+</sup> and CD8<sup>+</sup> cells belonging to different subjects, and in all cases the activity was highest with 1 mM Ca<sup>2+</sup>; increasing Ca<sup>2+</sup> concentration to 10 mM inhibited enzyme activity. The addition of 1 mM Zn<sup>2+</sup> led to inhibition of the activity, even when 10 mM Ca<sup>2+</sup> was present. DNA fragmentation was also measured in the absence of Ca<sup>2+</sup> at pH 8, 7.5, 7, 6, and 5 (Fig. 4). DNase activity associated with granules of CD4<sup>+</sup> or CD8<sup>+</sup> cells from different subjects increased with acidification of the medium; in all cases the activity was up to three times higher at pH 5 than at pH 8. In the absence of the enzyme and at the indicated pH values no spontaneous degradation of DNA was observed; however, at pH 4.7 DNA spontaneous degradation took place, and testing with the enzyme was not performed under those conditions. Fig. 4(A) shows the DNA fragmentation effected by CD4<sup>+</sup> cell granule associated

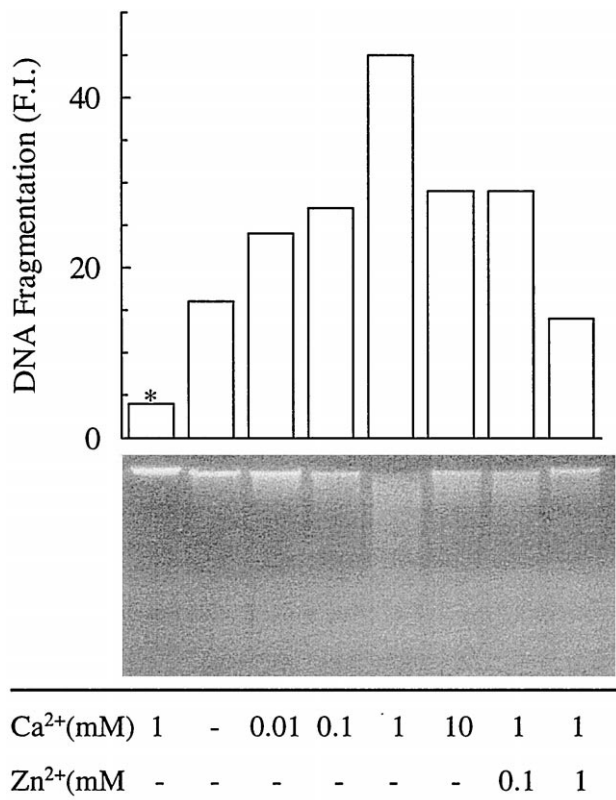


Fig. 3. Cation dependence of DNase. Protein extracts were incubated with genomic DNA for 12 h in 10 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 50 mM NaCl and the corresponding concentrations of divalent cations. Results are expressed as “fragmentation index” (F.I.) and correspond to a representative result of seven independent experiments. \* DNA incubated in buffer with optimal concentration of Ca<sup>2+</sup> but without protein extract (negative control).

DNase from a GD patient. Fig. 4(B) shows the effect of pH on a DNase activity belonging to CD4<sup>+</sup> cells from a PBC patient; in view of the high activity of that extract, when tested at pH 7.5, the incubation period was limited to 12 h (Fig. 4(B)). Fig. 4(C) shows the DNA fragmentation at low pH values with protein extracts of granules obtained from CD8<sup>+</sup> lymphocytes belonging to a MS patient. Since the medium used to detect the DNase activity contained DTT, we checked the possibility that this reducing agent might affect the enzyme under study, as it is the case with other DNases. No changes in the DNase activity from CD4<sup>+</sup> or CD8<sup>+</sup> cells granules were observed if incubations were carried out in the ab-

sence of DTT at pH 7.5 or pH 5. It was also seen that in the absence of this reducing agent this DNase activity was Ca<sup>2+</sup>-dependent. Therefore, this granule associated DNase activity from CD4<sup>+</sup> and CD8<sup>+</sup> cells was Ca<sup>2+</sup>-dependent, inhibited by Zn<sup>2+</sup>, and higher at low pH.

To find out whether different DNases were present in protein extracts from granules belonging to T lymphocytes and in order to determine their molecular weights, nucleases were visualized on the gels with the SDS-PAGE technique [26]. Protein extracts from secretion granules or from granule-free cytosol fraction belonging to T lymphocytes were separated by SDS-PAGE in gels containing DNA. After incubating in an appropriate buffer, spots with DNase activity appear as dark bands on a fluorescent background when ethidium bromide is added and gels are placed under UV light. A commercial 31 kDa DNase I (Sigma, St. Louis, Missouri) was used as reference control, and protein standards (Dalton Mark III L, Sigma) as molecular weight reference. Photographs of gels, after the electrophoresis of granule and granule-free cytosol extracts from CD4<sup>+</sup> lymphocytes belonging to a GD patient, are presented in Fig. 5. Identical results were obtained with a series of equivalent extracts of CD4<sup>+</sup> or CD8<sup>+</sup> from 7 different patients. When native double-strand DNA was used as substrate (Fig. 5(A)), one band of approximately 66 kDa appeared both with granule or granule-free cytosol extracts (lanes 1 and 2); a 31 kDa commercial DNase I, used as reference, was loaded on lane 3. However, a drastic decrease in the intensity of the bands was observed at the location corresponding to the 66 kDa DNase (Fig. 5(B)) when denatured DNA (previously heated at 100°C for 10 min) was present in the gel; under these conditions a new band appeared at approximately 31 kDa in the lane of the granule-free cytosolic extract (lane 2); an intense band was also visible at the location of the commercial DNase used as reference (lane 3). Therefore, the electrophoresis showed that granules contained a 66 kDa DNase preferentially fragmenting double-strand DNA, whereas they lacked the 31 kDa DNase, preferentially fragmenting single-strand DNA, which appeared in the granule-free cytosolic extract. Identical results were obtained when DTT was not added to the incubation medium used to carry out the DNase reaction on the gel.

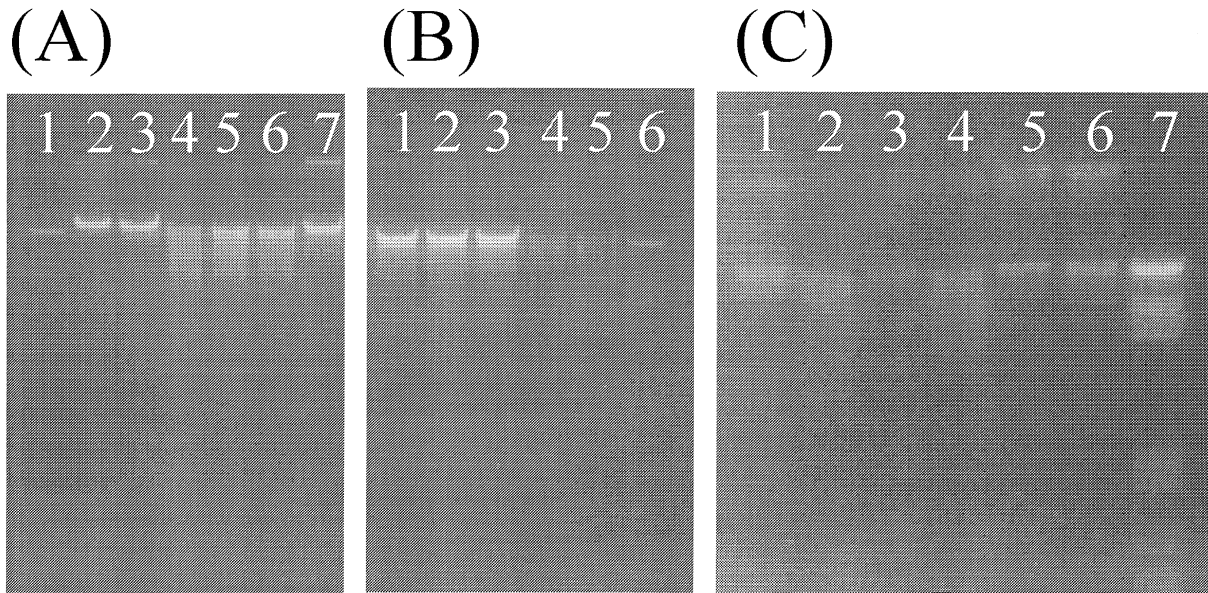


Fig. 4. Effect of pH on granule associated DNase. Protein extracts were incubated with genomic DNA in 10mM MgCl<sub>2</sub>, 50mM NaCl and the corresponding buffers at the indicated pH values. DNA fragmentation is representative of 10 independent experiments. DNA incubated at the indicated pH values without protein extract was used as negative control. (A) Granule DNase from T4 cells belonging to a GD patient incubated for 24 h. Lane 1, Standard DNA; Lanes 2 and 3, DNA incubated in pH 8 and 5 buffers respectively; Lanes 4–7, DNA incubated with protein extract at pH 5, 6, 7 and 8 respectively. (B) Granule DNase from T4 cells belonging to a PBC patient incubated for 12 h. Lanes 1 and 2, DNA incubated in pH 5 and 4.7 buffers respectively; Lanes 3–5, DNA incubated with protein extract at pH 8, 6 and 5. Lane, Standard DNA. (C) Granule DNase from T8 cells belonging to a MS patient incubated for 24 h. Lanes 1, 3 and 5, DNA incubated in pH 5, 6 and 7.5 buffers respectively. Lanes 2, 4 and 6 DNA incubated with protein extract in pH 5, 6 and 7.5 buffers respectively.

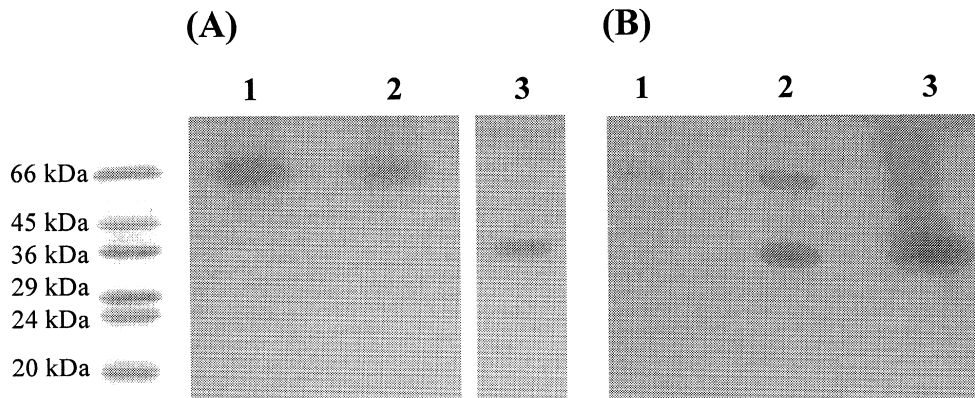


Fig. 5. DNase detection in SDS-PAGE. Protein extracts from granules or cytosol belonging to T lymphocytes with DNase activity were run in gels containing double-strand (A) or single-strand DNA (B). Lane 1, granule protein extract (20 μg); Lane 2, cytosolic protein extract (20 μg); Lane 3, reference DNase I (0.5 μg).

Table 1  
Mononuclear cell subpopulations in PBC, MS, GD and healthy donors

Cell population	HD	PBC	MS	GD
CD4 <sup>+</sup> (%PBMC)	37 ± 10	32 ± 13	29 ± 15	47 ± 8
CD8 <sup>+</sup> (%PBMC)	20 ± 3	15 ± 10	14 ± 7	17 ± 6
CD4 <sup>+</sup> CD45RA <sup>+</sup> (% of CD4 <sup>+</sup> )	45 ± 8	20 ± 6 <sup>a</sup>	30 ± 9 <sup>a</sup>	54 ± 14
CD4 <sup>+</sup> CD45RO <sup>+</sup> (% of CD4 <sup>+</sup> )	38 ± 16	42 ± 21	38 ± 16	31 ± 9
CD8 <sup>+</sup> CD11b <sup>+</sup> (% of CD8 <sup>+</sup> )	33 ± 10	42 ± 13	53 ± 12 <sup>a</sup>	34 ± 11
CD4 <sup>+</sup> CD45RO <sup>+</sup> /CD4 <sup>+</sup> CD45RA <sup>+</sup>	0.9 ± 0.5	2.5 ± 1.1 <sup>a</sup>	1.3 ± 0.7	0.8 ± 0.4

Results are expressed as percentage of positive cells for the indicated surface markers.

<sup>a</sup> Significantly different from control.

### 3.3. Phenotype of T subsets and its correlation with DNase activity

Table 1 summarizes the results obtained by phenotypic analysis of PBMC from subjects of the 4 groups studied. Several differences in the functional T cell subsets from patients were observed when compared with those present in healthy donors. In PBC and MS the proportion of CD4<sup>+</sup> cells with CD4<sup>+</sup>CD45RA<sup>+</sup> phenotype, naive cells whose major immunoregulatory function involves suppression, was significantly

lower than that found in healthy controls. In GD the proportion of CD4<sup>+</sup> within the PBMC population was strikingly high; therefore, the number of CD4<sup>+</sup>CD45RA<sup>+</sup> lymphocytes, corresponding to naive suppressor cells, as well as the number of CD4<sup>+</sup>CD45RA<sup>-</sup> lymphocytes, corresponding to helper cells inducers of cytotoxicity, were higher than in healthy donors. The proportion of CD4<sup>+</sup>CD45RO<sup>+</sup>, memory and cytotoxic cells, within the CD4<sup>+</sup> cells population was slightly higher in PBC and lower in GD; the number of CD4<sup>+</sup> within

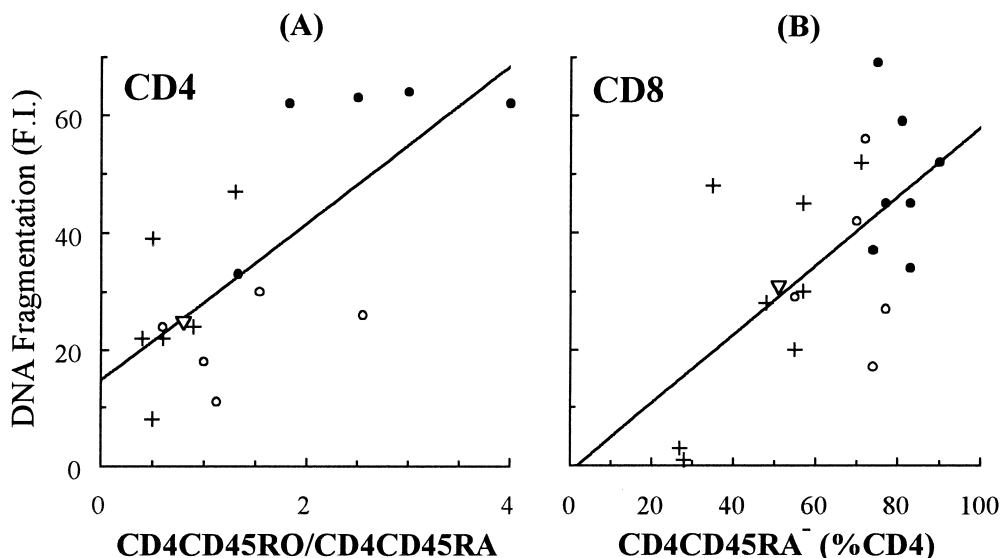


Fig. 6. Correlation between CTL granule DNase activity and CD4<sup>+</sup> subsets from peripheral blood. All the values corresponding to the different patients of each disease are represented: (●) PBC, (○) MS, (+) GD. In the case of healthy donors (HD), symbol (▽) indicates the mean of fifteen different individuals. (A) Correlation ( $p = 0.008$ ) between DNase activity associated with granules from CD4<sup>+</sup> cells and the ratio CD4<sup>+</sup>CD45RO<sup>+</sup>/CD4<sup>+</sup>CD45RA<sup>+</sup>. (B) Correlation ( $p = 0.02$ ) between DNase activity from granules of CD8. DNase activity is expressed as fragmentation index (F.I.) as defined in Section 2



the entire PBMC population was practically identical in PBC, GD and healthy donors, while it was slightly reduced in MS patients.

The differences in DNase activity and lymphocyte phenotype observed prompted us to look for possible correlations. We found that there was a direct correlation between the ratio of  $CD4^+CD45RO^+$ / $CD4^+CD45RA^+$  and DNase activity in T4 granules (Fig. 6(A)). This result is consistent with functions associated with T4 cell subsets: cytotoxic activity with  $CD4^+CD45RO^+$  memory cells and induction of suppression with  $CD4^+CD45RA^+$  cells. When  $CD8^+$  DNase activity was analyzed, a correlation between this activity and the percentage of  $CD4^+CD45RA^-$  cells (Fig. 6(B)) and between IL-2 secreted by these  $CD4^+$  helper cells (data not shown) was observed. These results are in agreement with the role of helper  $CD4CD45RA^-$  cells as cytotoxic-inducers of  $CD8^+$  and helper cells. In contrast, no correlation was found between DNase activities of T4 or T8 cells and the different T8 subsets.

In the 3 groups of patients the percentage of  $CD8^+$  within PBMC was slightly lower than that observed in healthy donors. However, in the patients with MS or PBC a selective enrichment in  $CD8^+CD11b^+$  lymphocytes, suppressor cells, within the  $CD8^+$  population was observed. On the other hand, the number of  $CD8^+CD11b^-$ , cytotoxic T8 lymphocytes, was lower in MS patients. Preliminary results obtained in our laboratory show that a small proportion of  $CD4^+$  cells expressed TIA-1 (approximately 10%), in contrast with the high proportion of  $CD8^+$  which expressed this antigen. This proportion was especially high in MS patients (70%).

#### 4. Discussion

The present report shows for the first time that both  $CD4^+$  and  $CD8^+$  lymphocytes from patients with PBC, and some with GD, contain proteins with DNase activity in secretion granules (Figs. 1 and 2). This granule associated DNase corresponded to a  $Ca^{2+}$  dependent 66 kDa protein (Fig. 3) more active at acid pH (Fig. 4), and also more active when double-strand DNA was used as substrate incorporated to the gel (Fig. 5(A)); it was, therefore, different from a 31 kDa DNase I, which was more active

with single-strand DNA (Fig. 5(B)); it was also distinguishable from other nucleases described as Type II DNases judging by its molecular weight or inhibition by  $Zn^{2+}$  ions [27,28].

Although the function of this DNase activity linked to lymphocytic granules is not yet clear, our data suggest that it might be another cytolytic factor; this would be in agreement with the idea that the expression of enzymes contained in secretion granules could be associated with the cytolytic capacity of T cells [29], and with the idea that the cytolytic mechanism of CTL could be related to an active transfer of a DNA fragmenting cytotoxic factor into target cells [30]. A possible involvement of the DNase activity, now being described, not only in the DNA fragmentation induced by CTL on target cells, but also in the apoptosis of CTL themselves, could be suggested. This idea is consistent with the observation that an increase in DNA fragmentation takes place in thymocytes, in parallel with an increase in calcium ions and chelation of intracellular zinc [31–33], or in CTL, as a consequence of intracellular acidification [34]. Furthermore,  $CD8^+$  CTL granule components, such as the TIA-1 RNA-binding proteins, have been described as part of a molecular cascade involved in Fas-mediated apoptosis [35,36].

On the other hand, it has been reported that  $CD4^+$  cytotoxic T cells induce apoptosis of HLA-II APCs [37,38], and it has been suggested that these cytotoxic cells could be involved in a direct destruction of target tissues because of the aberrant expression of HLA-II on the affected cells in autoimmune diseases [39–41]. Our results show that granule associated DNase both in  $CD4^+$  and  $CD8^+$  cells was enhanced in patients with PBC. This is consistent with a possible participation of infiltrating CTLs in the destruction of biliary ducts. DNase activity was lower in the GD patients studied than in the patients with PBC, possibly indicating a much deeper immunoregulatory disturbance in this latter disease. DNase activity was much lower in the MS patients studied, an observation consistent with the phenotype of their circulating lymphocytes: a low  $CD4^+CD45RO^+$ / $CD4^+CD45RA^+$  ratio and a low proportion of  $CD4^+CD45RA^-$ .

It has been described that in autoimmune diseases an imbalance between cytotoxic cells (or inducers of cytotoxicity) versus suppressors (or inducers of sup-

pression) occurs [8–11], [42,43]. Our data are also consistent with this idea. CTL cytotoxicity present in the patients studied and manifested by the presence of granule associated DNase (Figs. 1 and 2) could be due to an imbalance between activation of cytotoxicity and induction of suppression. Cytotoxic capacity of T4 cells, although not fully understood, is commonly associated with cells sharing the CD45RO<sup>+</sup> memory phenotype, and that of T8 cells with the CD11b<sup>-</sup> phenotype [44–46]. The correlation between CD4<sup>+</sup> DNase activity with the ratio of memory cells with cytotoxic potential (CD4<sup>+</sup>CD45RO<sup>+</sup>) to inducers of suppression (CD4<sup>+</sup>CD45RA<sup>+</sup>), shown on Fig. 6(A), prompts us to suggest that the appearance of cytotoxic T4 lymphocytes could be due to a differentiation of CD45RA<sup>+</sup> cells, inducers of suppression, into CD45RA<sup>-</sup> cells, helpers and inducers of cytotoxicity, thus modifying the normal immune homeostasis permitting existing memory cells to develop their cytolytic potential. In agreement with this, no increase in the number of CD4<sup>+</sup>CD45RO<sup>+</sup> cells was observed even in cases when DNase activity was higher than usual. Moreover, it has been described that a loss in cells with suppressor–inducer function might contribute to the disease process by allowing the immune response to proceed without feedback suppression [47]. In PBC, where we found the highest DNase activity, the CD4<sup>+</sup>/CD8<sup>+</sup> cell ratio was 2.5, a value much higher than that of 0.9 usually found in healthy individuals. The increase in cytotoxicity could be explained by the small number of CD4<sup>+</sup>CD45RA<sup>+</sup> cells (Table 1). Besides, in the case of PBC the proportion of CD4<sup>+</sup>CD45RA<sup>-</sup> cells, inducers of cytotoxicity, within the CD4<sup>+</sup> lymphocyte population amounted to about 80%, against a 45% in healthy controls, figures easily calculated from data in Table 1. This fact is consistent with the high DNase activity in CD8<sup>+</sup> lymphocyte granules in PBC, corroborating the correlation observed between such an activity and the CD4CD45RA<sup>-</sup> subset (Fig. 6(B)). Similarly, in those GD patients with detectable DNase activity both in T4 and T8 granules the CD4<sup>+</sup>CD45RO<sup>+</sup>/CD4<sup>+</sup>CD45RA<sup>+</sup> ratio as well as the proportion of CD4<sup>+</sup>CD45RA<sup>-</sup> within the CD4<sup>+</sup> cell population were higher than in those cases when DNase activity was not detected (Fig. 6).

We suggest that the DNase present in T4 and T8 cell granules, which we have seen to be associated

with an imbalance of cytotoxicity versus suppression in lymphocytes from patients, plays a role in the underlying mechanisms operating in autoimmune diseases, possibly through an increase in the cytolytic potential of T lymphocytes.

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