

# Early T Cell Activation Correlates with Expression of Apoptosis Markers in Patients with End-Stage Renal Disease

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**Abstract.** End-stage renal failure (ESRF) and chronic hemodialysis (HD) induce a state of immunodeficiency that involves T cell-mediated responses. A decreased T cell number combined with a reduced T cell lifespan and an increased T cell activation might play a role in the immune impairment associated with ESRF and chronic HD. Increased T cell activation associated with immunodeficiency suggests that activated T cells may be driven to apoptosis. To test this hypothesis, CD3<sup>+</sup> T cell activation (CD69) and apoptosis (annexin V, CD95 (Fas), and DNA fragmentation) were analyzed in a case control study after blood draw sampling (*ex vivo*), in culture conditions, and after phytohemagglutinin or anti-CD3 stimulation. *Ex vivo* evaluation of T cells showed an increased number of activated CD69<sup>+</sup> T cells in chronic HD patients ( $142 \pm 5$  cells/mm<sup>3</sup>) compared with patients with ESRF ( $115 \pm 2$  cells/mm<sup>3</sup>,  $P = 0.04$ ) and controls ( $74 \pm 2$  cells/mm<sup>3</sup>,  $P = 0.0006$ ). These data were confirmed in culture conditions and after stimulation. Similarly, annexin V and CD95 (Fas)-positive T cells were more numerous in both patient groups than in controls, irrespective of the experimental conditions ( $P \leq 0.005$  for both markers), and their percentage was always

significantly higher in chronic HD patients than in patients with ESRF. The amount of DNA fragmentation was also significantly higher in the cultured resting T cells of chronic HD patients ( $37 \pm 3\%$ ) than in those of patients with ESRF ( $25 \pm 3\%$ ) and controls ( $20 \pm 2\%$ ) ( $P = 0.01$ ). Percentage of cultured resting T cells expressing both CD69 and annexin V markers was higher in chronic HD patients ( $17 \pm 4\%$ ) than in patients with ESRF ( $10 \pm 4\%$ ) and controls ( $6 \pm 2\%$ ), ( $P = 0.005$ ). After stimulation (phytohemagglutinin or anti-CD3), CD69<sup>+</sup> T cell apoptosis increased by 2.4-fold in chronic HD patients compared with 1.8-fold in patients with ESRF and only 1.2-fold in controls ( $P = 0.001$ ). T cells from chronic HD patients and patients with ESRF thus showed an aberrant state of early activation that contrasted with an increased proportion of annexin V and CD95 (Fas)-positive T cells engaged in apoptosis, as confirmed by DNA fragmentation. Increased susceptibility to early activated T cell apoptosis is not only associated with uremia, but is also enhanced by HD procedure. This may account for the T lymphopenia, progressive immunodeficiency, and increased infection risk seen in these patients.

Various abnormalities of the immune system have been demonstrated in patients with end-stage renal failure (ESRF) (1–3). Furthermore, the impaired cellular immune response observed in uremic patients does not seem to be improved once hemodialysis (HD) is started (4,5). Indeed, chronic HD patients have clinical evidence of impaired lymphocyte (Ly) function (6–8). This immunodeficiency results from the uremic state itself but it is also enhanced by therapy (*i.e.*, HD) (1,2).

The mechanisms of immunosuppression in uremia and chronic HD are only partially understood. T cells seem to play a critical role (9–11), and it has been suggested that reduced T cell count (10) and impaired T cell proliferation (4) could account for immunodeficiency in the renal patients. We have

recently demonstrated that patients chronically hemodialyzed with biocompatible membrane showed elevated CD69 antigen compared with healthy controls (12). The induction of CD69 is an early biochemical event preceding T cell proliferation in subjects with normal kidney function. However, it is generally agreed that chronic HD patients show low T cell proliferative activity (4). These apparently contradictory data led us to address the question of the role played by activated CD69<sup>+</sup> T cells in patients with ESRF and in chronic HD patients. An attractive hypothesis is that CD69<sup>+</sup> T cells are driven into a cell death program because CD69 can be associated with cell division or cell apoptosis (13).

Apoptosis is a highly regulated process by which cells undergo programmed cell death leading to removal by phagocytosis (14,15). Recent studies suggest that in uremic patients, peripheral blood mononuclear cells, including T cells and monocytes, undergo accelerated apoptosis (16,17). However, little is known of T cell apoptosis, its magnitude, or its contribution to T cell dysfunction in patients with ESRF and in chronic HD patients. We undertook this study to evaluate T cell function in chronic HD patients and non-HD patients with

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ESRF compared with healthy controls, with special emphasis on the expression of early activation (CD69) and apoptotic (annexin V and CD95 (Fas)) markers by T cells under resting conditions and on mitogenic stimulation.

## Materials and Methods

### Patients and Controls

The study groups consisted of 18 patients with ESRF undergoing chronic HD for at least 6 mo before the study, of 8 patients with ESRF who were not hemodialyzed, and of a control group including 17 healthy subjects without renal impairment or any other disease and not taking medication. In this article, we use the term “patients with ESRF” to designate non-HD patients presenting end-stage renal disease with creatinine clearance between 10 to 15 ml/min that had to remain stable for at least 3 mo before the trial. All creatinine clearances were assessed according to the Cockcroft-Gault formula (18). Baseline characteristics of the three subjects groups are shown in Table 1. Only nonsmokers were enrolled in the study. Patients with acute or chronic infection, autoimmune disease, cancer, or previous radiation therapy, and patients receiving antiviral, cytostatic, steroidal anti-inflammatory or immunosuppressive medication were excluded from the study. Patients were negative for circulating hepatitis B antigen, hepatitis C antibody, and HIV. They had no active liver disease. No subject had undergone nephrectomy. All chronic HD patients had been dialyzed via the same membrane (polyamide) for at least 6 mo and had no significant residual renal function. The dose of dialysis (three times per week) remained constant according to the equilibrated Kt/V (eKt/V) urea formula. The normalized protein catabolic rate (expressed as gram per kilogram per day) was controlled before trial. All patients were in a stable anticoagulant state.

Medications, when needed, included antihypertensive treatment, orally administered antidiabetic therapy or insulin, vitamin D, phosphate, and potassium-exchanging resins. Intravenous iron therapy was administered according to the National Kidney Foundation Dialysis Outcomes Quality Initiative guidelines (19). Baseline serum ferritin levels were 200 to 600  $\mu\text{g/L}$  in all groups studied. During the study period, both patient groups were on erythropoietin therapy (89% chronic HD patients and 88% patients with ESRF). Treatment was adapted to maintain hemoglobin of 120 g/L. No subject had received blood transfusion during the last 6 mo before the study began. Informed consent was obtained from each participant according to the Helsinki Declaration of 1975, as revised in 1983. The study protocol was approved by the local ethics committee.

### Study Design

We assessed peripheral blood leukocyte, Ly, and CD3<sup>+</sup> T cell counts, a T cell activation marker (CD69), two apoptosis markers (annexin V and CD95 (Fas)), and T cell DNA fragmentation in chronic HD patients, non-HD patients with ESRF, and healthy subjects. The case control study design was chosen because this trial sought to evaluate the effects of uremia and chronic HD on T cell immunity.

### HD Therapy

Two different dialysis monitors, Integra (Hospal, Lyon, France) or AK-200 (Gambro Dialysatoren, Hünenberg, Switzerland), and a single-use, steam-sterilized, synthetic high-flux polymer (polyamide) hollow-fiber membrane (Polyflux S17, Gambro Dialysatoren, Hechingen, Germany) were used. The final concentration of dialysate was as follows: sodium 138 mmol/L, potassium 2.0 mmol/L, chloride

Table 1. Baseline clinical and laboratory characteristics of the study population<sup>a</sup>

Characteristics	Chronic HD Patients	Patients with ESRF	Controls
No. of patients	18	8	17
Age (yr)	51 $\pm$ 6 <sup>b</sup>	50 $\pm$ 5 <sup>b</sup>	49 $\pm$ 4 <sup>b</sup>
Gender (M/F)	9/9	4/4	8/9
Primary kidney diseases			
hypertensive nephroangiosclerosis	6	2	
diabetic nephropathy	4	2	
ischemic nephropathy	3	2	
ADPKD	3	1	
analgesic nephropathy	2	1	
Creatinine clearance (ml/min) <sup>c</sup>	5.1 $\pm$ 3.9 <sup>d</sup>	12.5 $\pm$ 2.1 <sup>d</sup>	96.8 $\pm$ 3.9 <sup>d</sup>
C-reactive protein level (mg/ml)	4 $\pm$ 1 <sup>b</sup>	4 $\pm$ 2 <sup>b</sup>	3 $\pm$ 2 <sup>b</sup>
iPTH level (pg/ml)	158 $\pm$ 42 <sup>d</sup>	148 $\pm$ 35 <sup>d</sup>	72 $\pm$ 5 <sup>d</sup>
Erythropoietin dosage (IU/kg per wk)	86 $\pm$ 12 <sup>e</sup>	82 $\pm$ 15 <sup>e</sup>	
Duration of HD (mo)	44 $\pm$ 13		
eKt/V	1.38 $\pm$ 0.08		
nPCR	1.32 $\pm$ 0.09		

<sup>a</sup> Values are given as mean  $\pm$  SD unless indicated otherwise. HD, hemodialysis; ESRF, end-stage renal failure; ADPKD, autosomal dominant polycystic kidney disease; iPTH, intact parathyroid hormone; nPCR, normalized protein carbolic rate.

<sup>b</sup>  $P > 0.05$  (ANOVA).

<sup>c</sup> Cockcroft-Gault formula.

<sup>d</sup>  $P = 0.0001$  (ANOVA).

<sup>e</sup>  $P > 0.05$  (Mann-Whitney  $U$  test).

102.25 mmol/L, calcium 1.38 mmol/L, magnesium 0.25 mmol/L, bicarbonate 31 mmol/L, glucose 2 g/L, and osmolarity 296 mOsm/L. Polyvinyl chloride blood lines (Bioline, Hospal Dasco Modella, Italy) were used for all treatments. The blood flow rate was maintained between 250 to 350 ml/min, and the dialysate flow rate was 500 ml/min. The ultrafiltration rate was regulated to achieve the dry weight of the patient. The dialysis sessions lasted 4 h. In all chronic HD patients, a native arteriovenous fistula was used as vascular access. Heparin doses were individually titrated to increase the partial thromboplastin time by 25 to 50%.

Microbiologic status and endotoxin concentrations of all dialysis fluids were documented every month (Institut Central des Hôpitaux Valaisans, Sion, Switzerland). Microbiologic status was documented by special nutrient-poor culture techniques. As recommended by the Association for the Advancement of Medical Instrumentation, tryptic soy agar was used to evaluate bacterial yields of dialysate. The upper allowable bacterial limit for all dialysis fluids was  $10^2$  colony-forming units per milliliter. Dialysate endotoxin concentrations were monitored by use of the *Limulus* amoebocyte lysate assay with chromogenic method (Coatest, Kabi Diagnostica, Nyköping, Sweden; sensitivity limit, 0.005 endotoxin units/ml). A well maintained reverse osmosis system, frequent disinfections of the entire flow path, and the introduction of ultrafiltration before the dialyzer provided sterile and pyrogen-free fluids (less than  $10^{-4}$  colony-forming units per milliliter and less than 0.125 endotoxin units per milliliter).

### Cell Preparations and Culture Conditions

All blood samples (27 ml) for T cell cultures were collected in heparin-coated tubes. In chronic HD patients, blood samples were drawn before the dialysis session through the arterial vascular access. Blood from patients with ESRF and healthy controls was drawn by venipuncture.

Peripheral blood leukocytes were isolated by standard techniques by use of Ficoll-Hypaque gradient density (density = 1077 g/L) centrifugation at 1000 rpm for 30 min at 20°C (Pharmacia LKB, Uppsala, Sweden). T cells were incubated on plastic tissue culture plates at 37°C for 1 h to allow monocytes to adhere, and nonadherent cells were passed over human T cell enrichment columns (R&D Systems, Minneapolis, MN) by use of high-affinity negative selection. The column-passed cells contained more than 95% CD3<sup>+</sup> T cells, as assessed by immunofluorescence that used anti-CD3 monoclonal antibody (mAb) (Immunotech Instrumentation Laboratory, Schlieren, Switzerland). Contamination with CD14<sup>+</sup> cells (monocytes) and CD19<sup>+</sup> cells (B cells) was less than 2%. All maneuvers were performed under strict sterile conditions.

Cell analysis was performed *ex vivo* within 30 min after blood was drawn. Cell cultures (without mitogen as resting T cells) were incubated in quadruplicate for 20 h in 96-well flat-bottomed plates (Nunc, Life Technologies, Paisley, Scotland).

### Phenotypic Cell Analysis

Cell analysis from chronic HD, patients with ESRF, and controls was performed by EPICS XL-MCL flow cytometer (Coulter Instrumentation Laboratory, Lausanne, Switzerland). We used fluorochrome-labeled mAb with their appropriate CD designations against CD3 (total T cells) and CD69 (early-activated T cells) (Immunotech Instrumentation Laboratory) in triple-color flow cytometry as described previously (20). Combinations of murine mAb directly conjugated to FITC, phycoerythrin, or phycoerythrin cyanine 5 were used according to manufacturer's instructions (Immunotech Instrumentation Laboratory) (20,21). The flow cytometer was calibrated with

flow-count beads, and results were analyzed with System II software (all from Coulter Instrumentation Laboratory). Ly gating was in general performed by the software on the basis of forward-scatter, side-scatter, and CD3 staining characteristics, but also manually when indicated. Results were expressed as a percentage of the total T cells (CD3<sup>+</sup>). A minimum of  $1 \times 10^5$  cells was studied in the Ly region.

### T Cell Stimulation In Vitro

For stimulation assays,  $1 \times 10^6$  T cells from both patient groups and controls were stimulated with 10 µg/mL purified phytohemagglutinin (PHA) (Murex, HA 16, Wellcome, Dartford, UK) or soluble monoclonal antibodies to CD3 (anti-CD3) (10 ng/well) (Sigma, St. Louis, MO) for 48 h at 37°C. Then, [<sup>3</sup>H]-thymidine ( $3.7 \times 10^4$  Bq/well) was added. Eight hours later, cells were harvested and measured in a liquid scintillation β-counter (Beckman LS 5000 CE, Beckman-Coulter Instrumental, Nyon, Switzerland). Flow cytometry was used for phenotypic analysis.

### Apoptosis Measurement

Cell apoptosis was assessed by annexin V staining with the flow cytometric apoptosis detection kit (PharMingen, Becton Dickinson, Basel, Switzerland), and by CD95 (Fas) staining with a direct mAb and flow cytometry (EPICS XL-MCL flow cytometer, Coulter Instrumentation Laboratory). Annexin V detects phosphatidylserine (PS) expression on the outer surface of cells during early apoptosis phase (22,23), and the CD95 (Fas) antigen is a polypeptide that plays a role in the programmed sequence of events leading to apoptosis of various cell types, including Ly (24,25).

Once isolated, T cells were adjusted to  $1 \times 10^6$  cells/ml and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, filtered through a 0.2-µm filter); 5 µl annexin V FITC (PharMingen, Becton Dickinson, Basel, Switzerland) was added to 195 µl of cell suspension. After 10 min of incubation in the dark, cells were washed and resuspended in 190 µl of binding buffer and 10 µl of propidium iodine stock solution (20 µg/mL) (PharMingen, Becton Dickinson, Basel, Switzerland). Live cells were considered to be those cells that were negative for both dyes; dead cells were positive for both fluorochromes; and apoptotic cells were positive only for annexin V FITC and negative for propidium iodine. The degree of apoptosis was assessed by flow cytometry.

For staining with anti-CD95 mAb (anti-Fas), T cells were incubated with saturating concentration of directly conjugated anti-CD95 (anti-Fas) mAb FITC (PharMingen, Becton Dickinson). Cellular expression of CD95 (Fas) was then analyzed by flow cytometry. DNA fragmentation was assessed in CD3<sup>+</sup> T cells that expressed annexin V, CD 95 (Fas), CD69/annexin V, or CD69/CD95 (Fas). Quantification of DNA fragmentation was performed by determination of fractional solubilized DNA by diphenylamine dye and spectrofluorometric assay. The DNA was precipitated with 0.5 ml of 25% TCA and was incubated for 15 min at 90°C to hydrolyze the DNA. Each DNA sample was then supplemented with 160 µl of diphenylamine reagents (Sigma), and color was allowed to develop for 4 h at 37°C. A total of 200 µl of colored solution was transferred to a well of a 96-well flat-bottom enzyme-linked immunosorbent assay plate, and OD was determined by a spectrophotometer at a wavelength of 620 nm. Percentage DNA fragmentation was calculated as the ratio of diphenylamine fluorescence in the supernatant divided by the total fluorescence in the supernatant plus the pellet multiplied by 100.

Qualitative DNA fragmentation analysis into nucleosomal bands was detected by agarose gel electrophoresis as previously described (26). In brief, cell pellets were lysed in 10 mM Tris-HCl/10 mM ethylenediami-

netetraacetate/0.2% Triton X-100 and centrifuged ( $13,000 \times g$ ) for 10 min. Supernatants containing RNA and fragmented DNA were extracted with phenol, followed by phenol–chloroform–isoamyl alcohol. The DNA/RNA was precipitated with ethanol and dissolved in 15  $\mu$ l of 10 mM Tris/HCl/1 mM ethylenediaminetetraacetate. Samples were treated with 600  $\mu$ g/mL of RNase (Sigma) and electrophoresed in 1% agarose gels, and DNA was visualized under ultraviolet light after staining with ethidium bromide.

### CD69/Annexin V and CD69/CD95 (Fas) Coexpression

After surface membrane labeling with phycoerythrin-conjugated anti-CD69 mAb for 30 min at 4°C, T cells were incubated for 5 min at 37°C with 5  $\mu$ l FITC-conjugated annexin V or anti-CD95 (anti-Fas) and analyzed by flow cytometry. The saturating amount of anti-CD69 mAb (1:20) was determined in preliminary experiments by testing increasing concentrations of the mAb on  $10^6$  cells/ml until fluorescence intensity remained unchanged.

### Statistical Analyses

Unpaired data were analyzed nonparametrically with the Mann-Whitney *U* test. The Wilcoxon rank-sum test was used for paired data. Significance of the differences in peripheral blood leukocytes, Ly, T cell subsets, proliferation, activation, and apoptosis between the groups was calculated by ANOVA and Bonferroni's multiple-comparison test. Results are expressed as mean  $\pm$  SD, or as the median in the case of skewed distribution, and range as specified. Statistical significance was defined as  $P < 0.05$ .

## Results

### Leukocyte Count and Differential

The absolute leukocyte count showed no significant difference between the groups studied ( $P = 0.07$ ) (Table 2). In contrast, the Ly count showed statistically significant lower values in chronic HD patients ( $1869 \pm 231$  cells/mm<sup>3</sup>) than in patients with ESRF ( $2144 \pm 125$  cells/mm<sup>3</sup>,  $P = 0.03$ ) and controls ( $2421 \pm 206$  cells/mm<sup>3</sup>,  $P = 0.0007$ ). Within Ly subsets, the mean absolute T cells (CD3<sup>+</sup>) number was significantly lower in chronic HD patients ( $1305 \pm 138$  cells/mm<sup>3</sup>) than in patients with ESRF ( $1554 \pm 110$  cells/mm<sup>3</sup>,  $P = 0.01$ )

and controls ( $1905 \pm 251$  cells/mm<sup>3</sup>,  $P = 0.0004$ ). B cells and non B–non T cells showed also statistically significant differences between the groups ( $P = 0.02$  and  $P = 0.03$ , respectively).

### CD69 T Cell Activation Marker

To investigate T cell activation in chronic HD patients, patients with ESRF, and controls, the early T cell activation marker CD69 was used. Evaluation of *ex vivo* T cells showed statistically significant higher surface coexpression of CD69/CD3 in chronic HD patients ( $142 \pm 5$  cells/mm<sup>3</sup>) compared with patients with ESRF ( $115 \pm 2$  cells/mm<sup>3</sup>,  $P = 0.04$ ) and controls ( $74 \pm 2$  cells/mm<sup>3</sup>,  $P = 0.0006$ ) (Figure 1A). In resting T cell cultures, CD69<sup>+</sup> T cells were higher in chronic HD patients ( $181 \pm 9$  cells/mm<sup>3</sup>) compared with patients with ESRF ( $131 \pm 7$  cells/mm<sup>3</sup>) and controls ( $83 \pm 3$  cells/mm<sup>3</sup>,  $P = 0.0006$ ). After anti-CD3 stimulation, the number of CD69<sup>+</sup> T cells raised to  $1071 \pm 105$  cells/mm<sup>3</sup>,  $776 \pm 67$  cells/mm<sup>3</sup>, and  $336 \pm 34$  cells/mm<sup>3</sup> in chronic HD patients, patients with ESRF, and controls, respectively ( $P = 0.0007$ ) (Figure 1B). In each experimental condition tested, chronic HD patients demonstrated significantly higher early activation than patients with ESRF (*ex vivo* and cultured resting T cells,  $P = 0.04$ ; after PHA or anti-CD3,  $P = 0.01$ ).

### T Cell Apoptosis

We measured the expression of annexin V and CD95 (Fas) in chronic HD patients, patients with ESRF, and controls. Table 3 shows that *ex vivo*, a significantly higher percentage of T cells from chronic HD patients expressed annexin V and CD95 (Fas) ( $34.7 \pm 4.9\%$  and  $32.9 \pm 4.2\%$ , respectively) when compared with T cells from patients with ESRF (annexin V:  $24.0 \pm 6.8\%$ ; CD95 (Fas):  $22.8 \pm 5.6\%$ , both  $P = 0.02$ ) and controls (annexin V:  $18.3 \pm 3.9\%$ ; CD95 (Fas):  $18.1 \pm 2.8\%$ , both  $P = 0.0004$ ). After culture, percentages of apoptotic resting T cells evaluated in chronic HD patients, patients with ESRF, and controls showed the same differences between the

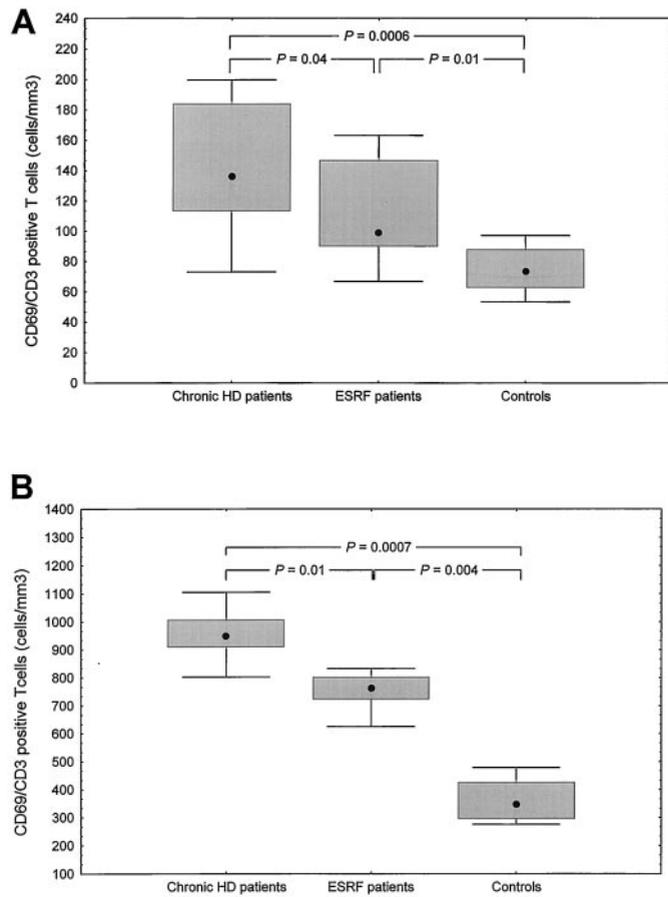
Table 2. Leukocyte count and differential in chronic hemodialysis (HD) patients, patients with end-stage renal failure (ESRF), and controls<sup>a</sup>

Test	Chronic HD Patients (n = 18)	Patients with ESRF (n = 8)	Controls (n = 17)	<i>P</i> <sup>b</sup>
Leukocytes (cells/mm <sup>3</sup> )	6,730 $\pm$ 1,156 <sup>h</sup>	7,054 $\pm$ 625 <sup>i</sup>	7,205 $\pm$ 1,274 <sup>h</sup>	0.07
Lymphocytes (cells/mm <sup>3</sup> )	1,869 $\pm$ 231 <sup>g</sup>	2,144 $\pm$ 125 <sup>i</sup>	2,421 $\pm$ 206 <sup>c</sup>	0.01
T cells (cells/mm <sup>3</sup> )	1,305 $\pm$ 138 <sup>e</sup>	1,554 $\pm$ 110 <sup>e</sup>	1,905 $\pm$ 251 <sup>d</sup>	0.005
B cells (cells/mm <sup>3</sup> )	142 $\pm$ 19 <sup>f</sup>	167 $\pm$ 15 <sup>f</sup>	143 $\pm$ 21 <sup>i</sup>	0.02
non B and non T cells (cells/mm <sup>3</sup> )	422 $\pm$ 27 <sup>i</sup>	422 $\pm$ 21 <sup>e</sup>	373 $\pm$ 29 <sup>e</sup>	0.03
Monocytes (cells/mm <sup>3</sup> )	442 $\pm$ 25 <sup>i</sup>	432 $\pm$ 19 <sup>e</sup>	396 $\pm$ 34 <sup>g</sup>	0.09
PMN (cells/mm <sup>3</sup> )	4,419 $\pm$ 302 <sup>i</sup>	4,478 $\pm$ 256 <sup>i</sup>	4,388 $\pm$ 452 <sup>i</sup>	0.4

<sup>a</sup> Values are given as mean  $\pm$  SD; PMN: polymorphonuclear leukocytes. Probability values displayed in the chronic HD patients column are for chronic HD patients *versus* ESRF patients, probability values in the ESRF patients column are for ESRF patients *versus* controls, and probability values in the controls column are for controls *versus* chronic HD patients.

<sup>b</sup> Comparison between the three groups (ANOVA).

<sup>c</sup>  $P = 0.0007$ ; <sup>d</sup>  $P = 0.0004$ ; <sup>e</sup>  $P = 0.01$ ; <sup>f</sup>  $P = 0.02$ ; <sup>g</sup>  $P = 0.03$ ; <sup>h</sup>  $P = 0.04$ ; <sup>i</sup>  $P > 0.05$ . Comparison between two groups (Mann-Whitney *U* test).



**Figure 1.** CD3<sup>+</sup> T cell count expressing CD69<sup>+</sup> early activation marker *ex vivo* (A) and after anti-CD3 stimulation (B) in chronic hemodialysis (HD) patients, patients with end-stage renal failure (ESRF), and controls. Box-and-whisker plots are used to represent the distributions. The bottom and the top of a box represent the 25th and 75th percentiles, and the dot in the box shows the median (50th percentile). Whiskers extend on either side of the box and aim to cover all observations, but they never exceed 1.5 times the height of the box (*i.e.*, the interquartile range). Any values outside the whiskers are plotted individually and are considered possible outliers. On a normal distribution, the box and whiskers cover roughly 99% of the population.

groups, whatever the marker used ( $P = 0.005$ ). After PHA or anti-CD3 stimulation, T cells from chronic HD patients expressed significantly higher surface concentration of annexin V and CD95 (Fas) than T cells from patients with ESRF and from controls (annexin V, both  $P = 0.0003$ ; CD95 (Fas), both  $P = 0.0004$ ).

The increased state of T cell apoptosis assessed by the annexin V and CD95 markers was confirmed by DNA analysis *ex vivo*, after culture, and after anti-CD3 stimulation. Figure 2A shows in every experimental condition the amount of DNA fragmentation of annexin V–positive T cells that was significantly higher in chronic HD patients ( $n = 8$ ) compared with patients with ESRF ( $n = 5$ ), ( $P = 0.007$ ) and controls ( $n = 8$ ), ( $P = 0.001$ ). As shown in Figure 2B, a characteristic fragmentation pattern of apoptosis was observed in DNA from annexin

**Table 3.** T cell apoptosis (percentage of annexin V and CD95 (Fas) positive T cells) in chronic hemodialysis patients, patients with ESRF, and controls analyzed *ex vivo*, in culture without activation (resting T cells) and after stimulation (phytohemagglutinin or anti-CD3)<sup>a</sup>

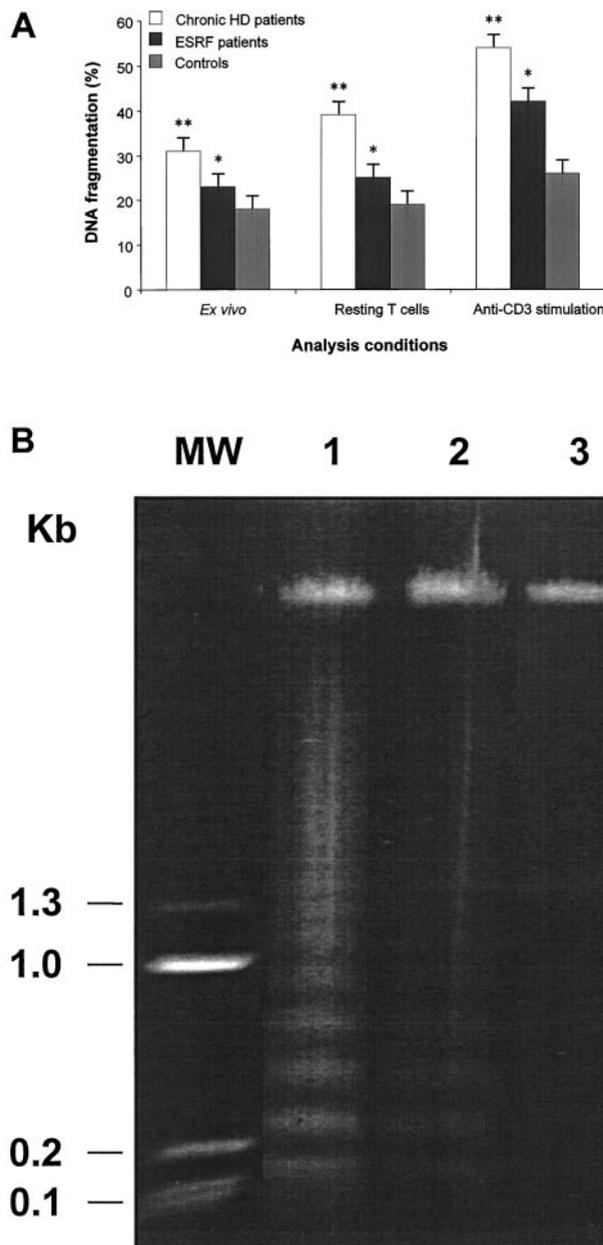
Medium	Chronic HD Patients ( $n = 18$ )		ESRF Patients ( $n = 8$ )		Controls ( $n = 17$ )		$P^b$	$P^b$	$P^b$
	% Annexin V Positive T Cells	% CD95 (Fas) Positive T Cells	% Annexin V Positive T Cells	% CD95 (Fas) Positive T Cells	% Annexin V Positive T Cells	% CD95 (Fas) Positive T Cells			
Ex vivo	34.7 ± 4.9 <sup>c</sup>	32.9 ± 4.2 <sup>c</sup>	24.0 ± 6.8 <sup>d</sup>	22.8 ± 5.6 <sup>d</sup>	18.3 ± 3.9 <sup>e</sup>	18.1 ± 2.8 <sup>e</sup>	0.002	0.003	0.003
Culture									
resting T cells	35.6 ± 3.6 <sup>c</sup>	34.8 ± 3.4 <sup>c</sup>	24.2 ± 6.6 <sup>f</sup>	24.0 ± 6.2 <sup>f</sup>	19.1 ± 3.7 <sup>e</sup>	18.5 ± 3.5 <sup>e</sup>	0.005	0.005	0.005
Stimulation									
PHA	53.8 ± 3.6 <sup>g</sup>	52.1 ± 3.8 <sup>g</sup>	42.2 ± 3.8 <sup>g</sup>	41.8 ± 4.1 <sup>g</sup>	28.8 ± 3.5 <sup>e</sup>	27.7 ± 3.8 <sup>e</sup>	0.0003	0.0004	0.0004
anti-CD3	54.2 ± 4.6 <sup>g</sup>	52.5 ± 4.2 <sup>g</sup>	44.4 ± 4.1 <sup>g</sup>	43.1 ± 3.8 <sup>g</sup>	30.0 ± 4.0 <sup>e</sup>	28.5 ± 3.6 <sup>e</sup>	0.0003	0.0004	0.0004

<sup>a</sup> Values are given as mean ± SD. They represent the number (%) of T cells (CD3<sup>+</sup>) expressing apoptosis markers (annexin V or CD95 (Fas)).

Probability values displayed in the chronic HD patients column are for chronic HD patients *versus* ESRF patients, probability values in the ESRF patients column are for ESRF patients *versus* controls, and probability values in the controls column are for controls *versus* chronic HD patients.

<sup>b</sup>  $P =$  comparison between the three groups (ANOVA).

<sup>c</sup>  $P = 0.02$ ; <sup>d</sup>  $P = 0.03$ ; <sup>e</sup>  $P = 0.0004$ ; <sup>f</sup>  $P = 0.05$ ; <sup>g</sup>  $P = 0.01$ ; comparison between two groups (Mann-Whitney  $U$  test).



**Figure 2.** (A) Percentage quantification (mean  $\pm$  SD) of DNA fragmentation of annexin V–positive T cells in 8 chronic hemodialysis (HD) patients, 5 patients with end-stage renal failure (ESRF), and 8 controls *ex vivo* (immediately after blood was drawn), after 20-h culture (resting T cells) and after anti-CD3 stimulation (positive control). \*,  $P = 0.01$ ; \*\*,  $P = 0.001$  versus controls. (B) Agarose gel analysis of T cell DNA fragmentation. Lane molecular weight (MW) is the size marker. Lanes 1 and 2 represent *ex vivo* T cell DNA isolated from a chronic HD patient and a patient with ESRF, respectively. Lane 3 represents *ex vivo* T cell DNA isolated from a control subject.

V–positive T cells of chronic HD patients and patients with ESRF, but not in DNA from T cells of the controls.

#### CD69/Annexin V and CD69/CD95 (Fas) Coexpression

Table 4 shows the results of CD69/annexin V and CD69/CD95 (Fas) T cell coexpression analyzed by flow cytometry.

In every condition tested, the percentage of activated CD69<sup>+</sup> T cells engaged in apoptosis, as shown by coexpression of annexin V or CD95 (Fas) and as confirmed by DNA fragmentation (Figure 3), was significantly higher in chronic HD patients than in patients with ESRF, and higher in patients with ESRF than in controls (Table 4). Differences were most amplified upon anti-CD3 or PHA stimulation. Figure 4 shows flow cytometry analysis of *ex vivo* CD69/annexin V coexpression on CD3<sup>+</sup> T cells in a representative chronic HD patient (A), a representative patient with ESRF (B), and a control subject (C).

#### Discussion

Given the central role of CD3<sup>+</sup> T cells in the immune response, we investigated T cell status by different markers of activation (CD69) and apoptosis (annexin V and CD95 (Fas)) in chronic HD patients and in patients with ESRF compared with controls.

Absolute numbers of CD3<sup>+</sup> T cells were decreased in patients with renal impairment (chronic HD patients and patients with ESRF). This observation is in agreement with previous reports (27,28). Although we have not addressed this question, T lymphopenia may correlate with the duration of dialysis dependency (27). T lymphopenia may be in part responsible for the uremia immunodeficiency, but profound abnormalities in T cell function were also found to exist (10,27,28). This led us to evaluate the T cell early-activation marker CD69. CD69 antigen is a phosphorylated cell surface protein and a member of the C-type lectin family, which is expressed early after T cell activation by PHA (29). T cell activation *via* CD69 usually results in upregulation of cytokines (interleukin-2, tumor necrosis factor alpha, and interferon gamma), which triggers T cell proliferation (13,29). We found that although T cell proliferative activity remained low in both groups of uremic patients (data not shown), numbers of CD69<sup>+</sup> T cells were significantly higher in chronic HD patients and in patients with ESRF than in controls. Our findings suggest that in chronic HD patients and patients with ESRF, a significantly high percentage of activated T cells ultimately do not proliferate but become apoptotic.

In apoptotic cells, the membrane phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to external cellular environment. Annexin V, a 35- to 36-kD, Ca<sup>2+</sup>-dependent phospholipid binding protein, has a high affinity for PS and binds to cells with exposed PS. Because externalization of PS occurs in the earlier stages of apoptosis, annexin V staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes (30,31). Therefore, staining with annexin V in conjunction with vital dyes such as propidium iodine allows identifying early apoptotic cells (annexin V positive, propidium iodine negative; data not shown) (17). We show that uremia *per se* markedly increases the number of annexin V–positive T cells, and that this effect is even greater when patients are chronically hemodialyzed.

Recent studies have indicated that uremic factors, including intracellular acidosis and alteration of Na<sup>+</sup>/H<sup>+</sup> antiporter set

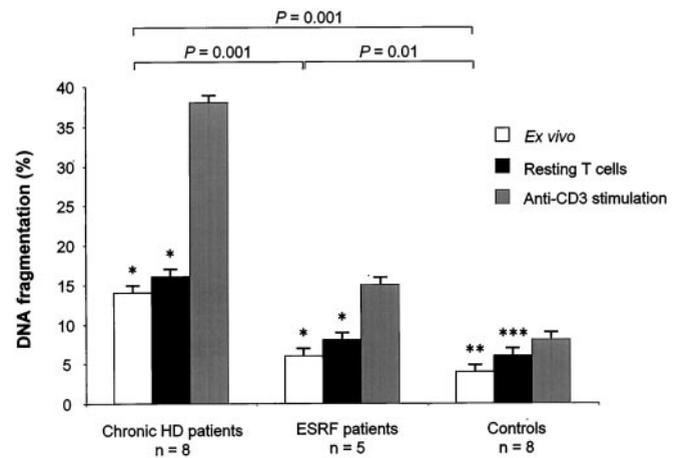
**Table 4.** CD69/annexin V and CD69/CD95 (Fas) coexpression in chronic hemodialysis (HD) patients, patients with end-stage renal failure (ESRF), and controls analyzed *ex vivo*, in culture without activation (resting T cells) and after stimulation (PHA or anti-CD3)<sup>a</sup>

Medium	Chronic HD Patients (n = 18)			ESRF Patients (n = 8)			Controls (n = 17)			P <sup>b</sup>	P <sup>b</sup>	P <sup>b</sup>
	% CD69/Annexin V Positive T Cells	% CD69/CD95 (Fas) Positive T Cells	% CD69/Annexin V Positive T Cells	% CD69/Annexin V Positive T Cells	% CD69/CD95 (Fas) Positive T Cells	% CD69/Annexin V Positive T Cells	% CD69/CD95 (Fas) Positive T Cells	% CD69/Annexin V Positive T Cells				
Ex vivo	15 ± 4 <sup>d</sup>	14 ± 4 <sup>d</sup>	8 ± 3 <sup>e</sup>	8 ± 3 <sup>e</sup>	5 ± 2 <sup>c</sup>	4 ± 2 <sup>c</sup>	0.0003	0.0003	0.0003			
Culture												
resting T cells	17 ± 4 <sup>e</sup>	16 ± 3 <sup>e</sup>	10 ± 4 <sup>f</sup>	9 ± 3 <sup>f</sup>	6 ± 2 <sup>c</sup>	5 ± 2 <sup>c</sup>	0.005	0.005	0.005			
Stimulation												
PHA	40 ± 8 <sup>d</sup>	39 ± 7 <sup>d</sup>	17 ± 6 <sup>d</sup>	16 ± 4 <sup>d</sup>	7 ± 2 <sup>c</sup>	6 ± 3 <sup>c</sup>	0.0003	0.0003	0.0003			
anti-CD3	41 ± 9 <sup>d</sup>	40 ± 8 <sup>d</sup>	18 ± 7 <sup>d</sup>	17 ± 6 <sup>d</sup>	7 ± 3 <sup>c</sup>	6 ± 2 <sup>c</sup>	0.0003	0.0003	0.0003			

<sup>a</sup> Values are given as mean ± SD. They represent the number (%) of CD69<sup>+</sup> T cells (CD3<sup>+</sup>) expressing apoptosis markers (annexin V or CD95 (Fas)). Probability values displayed in the chronic HD patients column are for chronic HD patients *versus* ESRF patients, probability values in the ESRF patients column are for ESRF patients *versus* controls, and probability values in the controls column are for controls *versus* chronic HD patients.

<sup>b</sup> Comparison between the three groups (ANOVA).

<sup>c</sup> P = 0.0003; <sup>d</sup> P = 0.01; <sup>e</sup> P = 0.02; <sup>f</sup> P = 0.03; comparison between two groups (Mann-Whitney U test).



**Figure 3.** Percentage quantification (mean ± SD) of DNA fragmentation of CD69<sup>+</sup>/annexin V-positive T cell performed in 8 chronic hemodialysis (HD) patients, 5 patients with end-stage renal failure (ESRF), and 8 controls *ex vivo* (immediately after blood was drawn), after 20-h culture (resting T cells) and after anti-CD3 stimulation (positive control). \*, P < 0.001; \*\*, P = 0.001; \*\*\*, P = 0.01 *versus* anti-CD3 stimulation.

points, are likely to be involved in T cell apoptosis (32,33). Thus, the uremic state is characterized by an increased expression of the early activation marker CD69 on T cells, contrasting with an increased expression of the apoptotic marker annexin V. We also demonstrated increased expression in the uremic state of another apoptosis marker, CD95 (Fas), a glycosylated cell-surface molecule of relative molecular masses of approximately 45,000 to 52,000 (335 amino acid residues) on CD3<sup>+</sup> T cells. Moreover, this study actually shows a strong association between early signs of T cell activation (CD69<sup>+</sup>) and T cell apoptosis (annexin V and CD95 (Fas) expression) in the three groups studied. Apoptosis was further confirmed by DNA fragmentation. This suggests that at least a proportion of activated CD69<sup>+</sup> T cells could succumb to apoptosis. In this respect, CD3<sup>+</sup> T cells that undergo spontaneous or dexamethasone-induced apoptosis have been shown to upregulate CD69, presumably as consequence of altered intracellular trafficking with subsequent CD69 accumulation on cell membranes (34).

Cytokines such as tumor necrosis factor alpha and interferon gamma can make cells more susceptible to apoptosis but are insufficient to induce apoptosis. We thus believe that in chronic renal failure and chronic HD, the effects of these cytokines combined with antigen-presenting cells (*i.e.*, monocytes and other peripheral blood leukocytes) are required for the CD95 (Fas) expression on CD3<sup>+</sup> T cells, hence making them susceptible to undergo apoptosis. Alternatively, we cannot rule out the possibility that the soluble form of CD95L (FasL) is present in the serum of people with chronic renal failure.

Our findings also demonstrate that more T cells from chronic HD patients than from patients with ESRF developed apoptosis. We suggest that T cell apoptosis in the context of high levels of the early activation marker CD69 may be attrib-

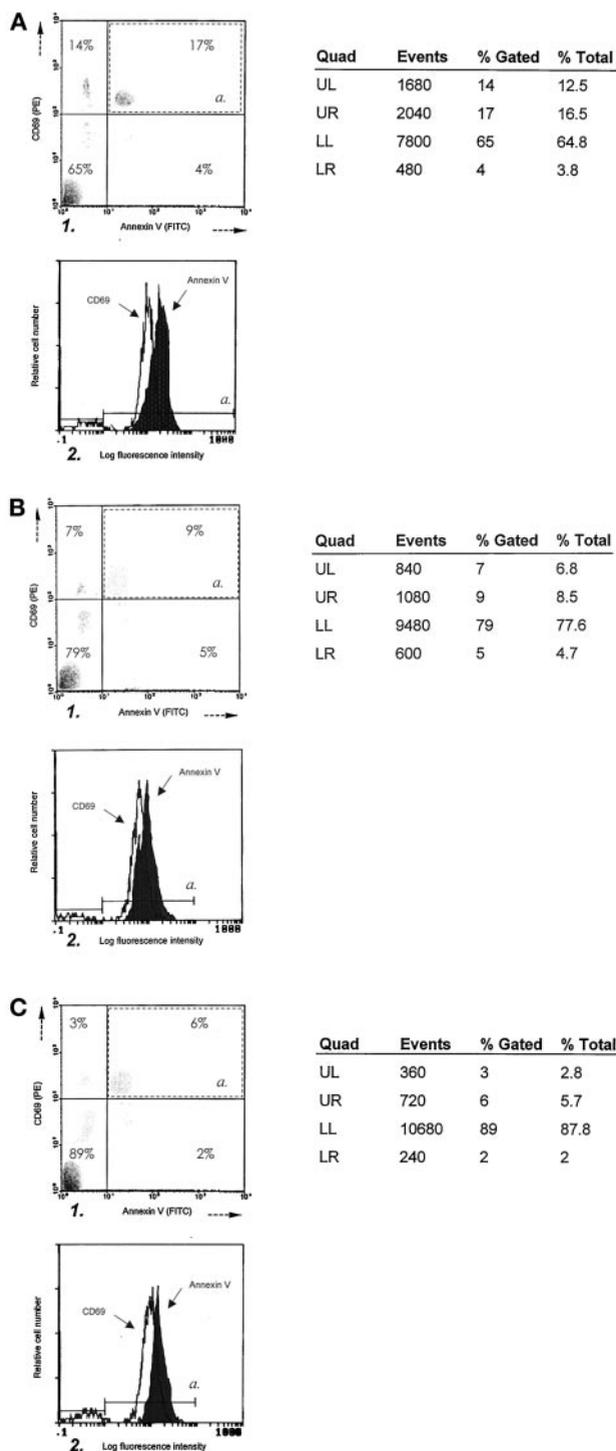


Figure 4. Flow cytometry analysis of CD3<sup>+</sup> T cells expressing both CD69 (phycoerythrin [PE])/annexin V (FITC) markers *ex vivo* in one representative chronic hemodialysis (HD) patient (A), one representative end-stage renal failure (ESRF) patient (B), and one control subject (C). Panels 1 show the distribution of CD3<sup>+</sup> T cell population in each patient studied. Horizontal and vertical lines indicate the threshold for the detection of antigen expression (*i.e.*, CD69 [PE]/annexin V [FITC]). Panels 2 show T cell frequency (relative cell number) that present CD69/annexin V coexpression (log fluorescence intensity). The *a* gate demarcates double positive (CD69/annexin V positive) populations. Quad, quadrants; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

utable to the intermittent contact between blood and the artificial surfaces of the extrinsic HD membrane and other components of the extracorporeal circuit or to oxidative stress or middle-sized molecules, which are more prominent in chronic HD patients (35–38). Other explanations for increased apoptosis can be reasonably excluded. The chronic HD patients recruited for the study showed no signs of iron overload or an ongoing immunologically active primary disease that could influence high T cell apoptosis. No chronically infected subject was included in the study. Similarly, we could rule out the role of endotoxins through the production of anti-inflammatory cytokines because endotoxin levels in the water purification system were low (39). Finally, the T cell preparation procedure eliminates monocytes, polymorphonuclear cells, and platelets that might play a role in T cell activation.

Because the chronic interaction of blood with the synthetic HD membrane leads to an early activation of T cells with a putative influence on cell survival (40,41), it was of interest to analyze apoptosis before and after the dialysis procedure. We found that in chronic HD patients, a single HD session induced a slight but significant increase (15 ± 3%) in the T cell population coexpressing *ex vivo* CD69 and annexin V or CD69 and CD95 (Fas) (data not shown). Thus, dialysis sessions could chronically stimulate programmed T cell death.

In summary, this study provides a rationale explanation to the finding of T cell immunodeficiency in uremic patients. Activation of T cell assessed by CD69 expression is associated with an increased expression of the apoptosis annexin V and CD95 (Fas) markers, suggesting that CD69<sup>+</sup> T cells are committed to apoptosis—hence the T lymphopenia and the deficiency of T cell responses in uremic patients. These anomalies are amplified once HD has been started. Further studies are required to define the T cell apoptosis regulatory mechanisms and biochemical processes involved in patients with ESRF and in chronic HD patients.

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