DUAL EFFECT OF METHYLPREDNSOLONE PULSES ON APOPTOSIS OF PERIPHERAL LEUKOCYTES IN PATIENTS WITH RENAL DISEASES

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It is well known that change in apoptosis may modulate the natural story of illness, and that many drugs may act through modulation of apoptosis, but the role of steroids in acting through apoptosis in different settings, including renal diseases, has still to be elucidated. We studied the *in vivo* effects of steroids by oral assumption (10 to 25 mg/deltacortene) or by intravenous pulses (300 to 1000 mg/dose) on apoptosis and cellular subsets of peripheral lymphocytes, by evaluating DNA-fragmentation and lymphocyte subsets in 79 subjects: 22 controls and 57 patients with various renal diseases (25 Lupus-GN, 19 membranous-GN (MGN), 6 rapidly progressive-GN (RPGN), 2 acute interstitial nephritis (AIN), 5 on chronic dialysis. Baseline apoptosis was present in 1/22 (4.5%) of controls, 3/25 (12%) SLE, 2/6 (33.3%) RPGN and 10/19 (52.6%) MGN. A significant decrease in CD3+CD8+ cell count and a significant increase of the CD3+CD4/CD3+CD8+ ratio were found in apoptosis-positive subjects. DNA fragmentation did not change after oral steroids, paralleling a 22 to 32% decrease in total lymphocytes. Following intravenous methylprednisolone pulses, a deeper drop of all lymphocyte subsets was observed, while DNA fragmentation turned from present to absent in 2 MGN, but not in 2 RPGN, and from absent to present in 1 ARF and 1 SLE, independently of the dosage. We demonstrated that the presence of apoptosis in renal diseases is associated with decreased CD3+CD8+ cell count. Furthermore, steroid intravenous pulses, besides inducing a profound decrease in lymphocyte subsets, do exert a dual effect on baseline leukocyte apoptosis, eventually leading to a reversal of baseline patterns, either turning from negative to positive or from positive to negative. Oral steroid therapy did not influence baseline apoptosis.

Apoptosis is the most common form of cell death in the organism (1-3). It is now clear that either “too little” or “too much” apoptosis may cause illnesses. For example, “too little” apoptosis might be involved in the increase of tumor mass (net result of uncontrolled proliferation and reduced cell death) and in autoimmune diseases such as Systemic Lupus Erythematosus (SLE), in which a reduced apoptosis may lead to an accumulation of malfunctioning lymphocytes responsible for the formation of autoantibodies (4). On the contrary, “too much” apoptosis has been found in some liver diseases and in AIDS, where lymphocyte apoptosis increases as the disease progresses (5).

Key words: apoptosis, steroid, lymphocyte subsets, primary glomerulonephritis, Systemic Lupus Erythematosus, vasculitis, membranous glomerulonephritis

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Induction of apoptosis is an extremely complex process involving a puzzle of interactions including the surface molecule named Fas/CD95 (which is expressed on activated lymphocyte and can trigger cell death following interaction with its ligands), a family of cysteine proteases, the caspases (responsible for the cellular changes that occur during apoptosis) and human major histocompatibility complex (MCH) class I molecule-specific gene such as the bcl-2proto-oncogene (6-7).

Glucocorticoids hormones have been implicated as regulators of T-lymphocyte growth and differentiation. They are apoptosis activators and induce T-cell death, but they can also counteract apoptosis activated by other stimuli, thus rendering the goal of identifying the mechanism by which steroids exerts specific immunosuppressive action a fascinating topic (8-10). At the same time, glucocorticoids have a major role in the treatment of renal diseases, but very few studies have addressed the role of steroids in modulating immune cells apoptosis in this setting .

The aim of our study is to evaluate: a) baseline apoptosis of peripheral white cells in patients with different renal diseases; b) the role of steroids given *per os* or intravenous infusion on apoptosis of peripheral white cells and on cellular subsets of peripheral lymphocytes of patients with different renal diseases, in a prospective study comparing these results with the results of a normal population assumed as control.

MATERIALS AND METHODS

Study population

The study was carried out on 79 subjects, 22 healthy volunteer donors and 57 patients with active and inactive renal diseases. The control population consisted of 22 healthy individuals, 13 women and 9 men, ranging from 20 to 53 years old (mean 32 ± 6 years).

The 57 patients (35 women, 22 males) with renal diseases included 25 SLE (22 women, 3 men, mean age 39± 10 years, follow-up from the onset of disease from 2 to 23 years, serum creatinine ranging from 0.6 to 2.8 mg/dL) 19 primary Membranous Glomerulonephritis (MGN) (9 women, 10 men, mean age 52±14 years, follow-up from the onset of disease from 6 months to 22 years, serum creatinine ranging from 0.9 to 1.3 mg/dL) 6 Rapidly Progressive Glomerulonephritis (RPGN) (2 women, 4 men, mean age 68.5±3.7 years, serum creatinine ranging from 1.4 to 5. 5 mg/dL), 2 acute interstitial nephritis (AIN) (one 72 year-old man, one 75 year-old woman, serum creatinine 6.2 and 5.6 mg/dL, respectively) and 5 patients with chronic renal failure on regular dialytic treatment (RDT) (1 woman, 4 men, mean age 55±15years, on regular treatment from 2 to 20 years).

Diagnosis of renal diseases, excluding that for patients on chronic dialysis, had been previously obtained by percutaneous renal biopsy. All the studies were performed in patients at different stages of their diseases, without interventional modification of the ongoing therapeutic protocols.

Among SLE patients, n 1 was in a flare-up phase on intravenous steroid pulses (1000 mg/day per three days), n 14 in a phase of mild activity (n 10 on oral steroid 10 to 25 mg/day plus azathioprine 1.5 mg/Kg/day and n 4 on steroid 10 mg/day plus hydroxychloroquine 300mg/week), and n 10 in complete remission without immunosuppressants.

Among patients with MGN, n 2 were in a flare-up phase on intravenous steroid pulses (500 to 1000 mg/day per three days), n 7 in a phase of mild activity (on oral steroid 5 to 30 mg/day) and n 10 were on remission, without immunosuppressants.

All the patients with RPGN (two Wegener disease, two Good-Pasture syndromes and two type III) were on activity phase, n 2 on intravenous steroid pulses (1000 mg/day per three days) and n 4 on oral steroid (50 mg/day) coupled with cyclophosphamide 2mg/Kg.

The two patients with immunoallergic AIN were given intravenous methylprednisolone pulses (500 to1000 mg/day per three days).

Cell preparation and flow cytometry

Blood was collected in EDTA, and a peripheral blood count was performed by standard automated analysis (Sisemx Toa Ne-8000). Flow cytometry was performed with a flow cytometer (Epics XL, Coulter Electronic Co., Hialeah, FLA, USA). Samples were centrifuged through Ficoll-Hypaque, interface cells were collected, and the cells washed with RPMI medium. Re-suspended cells (0.1 mL) were incubated for 20 minutes in an ice bath with 20 μL each of the designated antibodies at titered concentrations. (a) For sorting, re-suspended cells (usually 25 μL) were incubated with the corresponding monoclonal antibody CD3- ECD (peripheral T-cells), CD4-PE (helper-inducer T-cell), CD8-FITC (suppressor-cytotoxic T-cell), CD16-FITC (natural killer lymphocytes), CD19 –ECD (B-lymphocytes). After 20 minutes incubation, the cells were treated with Whole Blood Lysing Kit. Quality control was made by Coulter Cytotrol Control Cells.

Detection of apoptosis

DNA extraction and electrophoresis were performed as described elsewhere (11). In brief, peripheral blood

was collected in EDTA. After centrifugation, supernatants containing lymphocytes and monocytes were lysed in a hypotonic buffer (100 mM Tris-HCL pH 8.5, 5 mM EDTA, 0.2%SDS, 200 mM NaCl and proteinase K 100 µg/mL) for 1 hour at 50°C. DNA was extracted by phenol/chloroform and separated on a 1.5% agarose gel. After vortex, samples were centrifuged at 13000 x g for 5 min at room temperature. The pellet was collected and added to CH3COONA pH 5.2 2 volumes ethanol in ice 1/100 MgCl2, and then at –80°C for 12 hours. Next morning, after centrifugation, at 13000 g for 5 min a 4°C, the surnatant was re-suspended and a further washing in ethanol 70% was repeated. After elimination of the ethanol, the sample was re-suspended in 50 microl of TE (1 mMEDTA, 10 mMTris-HClpH 8.0).

Electrophoresis

In each sample, an addensant solution colouring 6X (0.25% v/v bromophenol, 0.25%v/v xhylenxyanole, 30% v/v glycerol in water) was diluted. The sample, the positive control and a marker of molecular weight (marker IX, Roche Molecular Biochemicals) were mounted on agarose gel 1.5%: 0.5 µg/ml of ethidium bromide, and underwent electrophoresis in TBE 1X (from TBE 10X.0.9M Tris-base, 0.9 boric acid, 100 mM EDTA, at 50 V for 40 minutes.

Positive controls

A sample from one healthy individual was processed as detailed above, divided into three Falcon tubes filled with saline and then irradiated at 600 rad (Co) with incubation at 37°C for 18 hours (12).

Protocol study

A count of cellular subsets of peripheral lymphocytes and evaluation of DNA apoptosis were performed in all subjects in the morning (h 8, baseline). Furthermore, the same analyses were repeated at h 12, in patients receiving both oral or iv steroid pulses four hours before (immediately after baseline evaluation) as well as in 10 controls who did not take any drug, in order to exclude circadian changes in apoptosis.

RESULTS

Baseline apoptosis

Baseline apoptosis was absent in patients with AIN and RDT, while it was demonstrated in 1/22 (4.5 %) controls, 3/25 of SLE (12%, one on remission,

Table I. *Lymphocytes subsets and clinical data in controls and patients with renal diseases, according to baseline apoptosis.*

	Controls n 22		SLE n 25		MGN n 19		RPGN n 6	
	apoptosis + n 1	apoptosis - n 21	apoptosis + n 3	apoptosis - n 22	apoptosis + n 10	apoptosis - n 9	apoptosis + n 2	apoptosis - n 4
CD3+ (x 10 ^{9}/l)	0.59	1.37±0.48	1.03±0.35	1.37±0.48	1.33±0.67	1.71 ±1.9	0.93±.16	1.71±0.55
CD3+CD4+ (x 10 ^{9}/l)	0.40	0.98±0.23	0.74±0.21	0.81±0.31	1.26±0.57	1.15±0.43	0.76±0.11	0.53±0.35
CD3+CD8 (x 10 ^{9}/l)	0.17	.57±0.24	0.27±0.12*	0.52±0.19	0.28±0.72*	0.61±0.22	0.15±0.06*	0.56±0.23
CD3+CD4+/ CD3+CD8 ratio	2.33	1.94±0.71	2.99±0.61†	1.60±0.45	4.37±2.68†	2.02±0.59	5.46±1.46†	2.06±0.72
HLA-DR+	0.40	0.42±0.32	0.13±0.06	0.24±0.15	0.27±0.06	0.37±0.11	0.24±0.05	0.22±0.11
Activated Lymphocytes	0.01	0.02	0.03±0.03	0.03±0.04	0.02±0.01	0.02±0.01	0.01±0.01	0.03±0.02
Acute phase	-	-	0	1/1	2/2	0/2	2/6	
Mild activity phase	-	-	2/14	12/14	3/6	3/6		
Remission	-	-	1/10	9/10	5/10	5/10		
RPGN category:	-	-						
Wegener							1	1
Good-Pasture							1	1
Type III								2

Abbreviations: SLE = Systemic Lupus Erythematosus
MGN = membranous Glomerulonephritis
RPGN = Rapidly Progressive Glomerulonephritis
** $p < 0.01$ apoptosis + vs apoptosis- †= $p < 0.001$ apoptosis + vs apoptosis-*

Table II. *Apoptosis and lymphocyte subsets in controls and in patients with renal diseases at 8 a.m and 12 a.m without therapy*

	Controls n 10		RDT n 5		SLE n 10		MGN n 10			
							Apoptosis - (n 4)		Apoptosis + (n 6)	
	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12
Apoptosis	absent	absent	absent	absent	absent	absent	absent	absent	present	present
Leukocytes(x 10^9/l)	6.98±1.31	7.86±1.63	6.94±2.12	6.53±2.48	6.62±1.18	7.33±1.97	6.98±0.74	7.91±0.83	5.36±0.95	5. 76±1.13
Lymphocytes(x 10^9/l)	2.17±0.43	2.52±0.81	1.62±0.94	1.30±0.39	2.03±0.47	1.74±0.60	2.53±0.35	2.65±0.42	1.67±0.68	1.42±0.60
CD3+ (x 10^9/l)	1.51±0.48	1.74±0.87	1.09±0.59	0.88±0.35	1.49±0.39	1.27±0.42	1.75±0.25	1.99±0.28	1.03±0.41	0.79±0.31
CD3+CD4+ (x 10^9/l)	0.93±0.27	1.11±0.51	0.72±0.35	0.60±0.25	0.9±0.25	0.74±0.28	1.02±0.12	1.16±0.11	0.90±0.58	0.58±0.29
CD3+CD8 (x 10^9/l)	0.55±0.23	0.61±0.37	0.35±0.24	0.27±0.11	0.57±0.13	0.48±0.21	0.69±0.14	0.80±0.21	0.23±0.06	0.15±0.02
CD3+CD4+/ CD3+CD8 ratio	1.80±0.36	1.94±0.45	2.37±1.0	2.27±0.73	1.58±0.30	1.64±0.47	1.50±0.19	1.51±0.29	3.63±2.2	3.81±1.72
HLA-DR+	0.31±0.1	0.38±0.18	0.20±0.17	0.14±0.08	0.27±0.09	0.26±0.13	0.39±0.09	0.40±0.08	0.27±0.08	0.28±0.07
Activated Lymphocytes	0.02±0.03	0.03±0.02	0.03±0.02	0.02±0.01	0.03±0.03	0.03±0.04	0.04±0.05	0.02±0.01	0.03±0.02	0.02±0.01
B Lymphocytes (CD 19+)	0.28±0.11	0.37±0.17	0.17±0.16	0.11±0.07	0.23±0.09	0.23±0.14	0.36±0.06	0.41±0.08	0.24±0.08	0.25±0.07
NK Lymphocytes (CD 16+)	0.34±0.15	0.32±0.17	0.32±0.24	0.26±0.72	0.23±0.14	0.21±0.15	0.24±0.22	0.28±0.22	0.25±0.17	0.24±0.21

Abbreviations: SLE = Systemic Lupus Erythematosus, MGN= membranous Glomerulonephritis, RPGN= Rapidly Progressive Glomerulonephritis

Table III. *Effects on apoptosis and lymphocyte subsets following oral steroid therapy in patients with renal diseases.*

	SLE n 11				MGN n 6				RPGN n 6			
	Apoptosis - (n 9)		Apoptosis + (n2)		Apoptosis - (n 5)		Apoptosis + (n 1)		Apoptosis - (n 4)		Apoptosis + (n 2)	
Apoptosis	h 8 *	h 12**	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12
	absent	absent	present	present	absent	absent	present	present	absent	absent	present	present
Leukocytes(x 10 ^{9}/l)	6.57±2.06	8.83±2.8	5.31±3.59	6.35±5.27	8.39±2.82	9.31±2.93	4.73	6.07	8.56±1.67	9.04±0.85	10.70±5.57	11.11±8.53
Lymphocytes(x10 ^{9}/l)	1.88±0.81	1.08±0.39	1.48±0.64	1.35±0.61	2.62±0.68	1.99±0.94	2.3 0	1.10	2.57±0.68	1.97±0.40	2.1±0.21	1.64±1.54
CD3+ (x 10 ^{9}/l)	1.47±0.44	0.81±0.39	1.13±0.44	0.92±0.24	1.87±0.64	1.46±0.82	1.52	0.69	1.71±0.55	1.30±0.35	1.45±0.16	1.24±1.15
CD3+CD4+ (x 10 ^{9}/l)	0.85±0.40	0.41±0.23	0.80±0.24	0.61±0.06	1.19±0.47	1.05±0.61	1.10	0.50	1.11±0.39	0.86±0.35	0.77±0.11	0.43±0.94
CD3+CD8 (x 10 ^{9}/l)	0.59±0.24	0.35±0.17	0.31±0.14	0.29±0.16	0.59±0.18	0.59±0.23	0.37	0.18	0.57±0.23	0.43±0.14	0.43±0.06	038±0.22
CD3+CD4+/ CD3+CD8 ratio	1.44±0.24	1.15±0.117	2.67±0.33	2.44±1.17	3.95±0.56	3.42±0.59	3.0	2.81	2.06±0.72	2.19±1.24	5.47±1.46	2.91±1.10
HLA-DR+	0.27±0.22	0.16±0.09	0.11±0.07	0.10±0.06	0.37±0.10	0.40±0.05	0.2	0.12	0.22±0.11	0.22±0.06	0.25±0.05	0.37±0.28
Activated Lymphocytes	0.05±0.06	0.04±0.05	0.04±0.04	0.04±0.04	0.03±0.03	0.02±0.01	0.02	0.02	0.4±0.02	0.04±0.02	0.02±0.01	0.02±0.01
B Lymphocytes (CD 19+)	0.18±0.14	0.11±0.006	0.08±0.03	0.05±0.1	0.33±0.11	0.37±0.04	0.16	0.11	0.18±0.09	0.18±0.04	0.21±0.01	0.31±0.26
NK Lymphocytes (CD 16+)	0.15±0.12	0.14±0.11	0.21±0.15	0.29±0.34	0.34±0.15	0.32±0.21	0.53	0.26	0.62±0.16	0.38±0.17	0.03±0.01	0.07±0.06

Abbreviations: SLE = Systemic Lupus Erythematosus, MGN= membranous Glomerulonephritis, RPGN= Rapidly Progressive Glomerulonephritis
**baseline, before oral prednisone therapy*
***four hours after oral prednisone therapy*

Table IV. *Effects on apoptosis and lymphocytes subsets following methylprednisolone pulses in patients with renal diseases.*

	SLE n 1 (1000mg)		IRA n 2				MGN n 2				RPGN n 2			
			n1 (500mg)		n1(300mg)		n1 (500mg)		n1(1000mg)		n1(1000 mg)		n1(1000 mg)	
	h 8*	h 12**	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12	h 8	h 12
Apoptosis	absent	present	absent	present	absent	absent	present	absent	present	absent	present	present	present	present
Leukocytes(x 10^9/l)	2.72	2.80	10.15	9.27	8.68	10.21	9.62	8.95	15.85	16.36	14.99	13.33	6.76	5.07
Lymphocytes(x 10^9/l)	1.04	0.54	0.85	0.38	1.69	1.05	2.92	1.01	0.94	0.82	2.04	0.61	1.06	0.55
CD3+ (x 10^9/l)	0.84	0.32	0.41	0.18	1.15	0.44	2.04	0.60	0.53	0.42	1.57	0.33	0.82	0.42
CD3+CD4+ (x 10^9/l)	0.64	0.22	0.28	0.14	0.78	0.23	1.05	0.29	0.34	0.28	1.06	0.19	0.69	0.28
CD3+CD8 (x 10^9/l)	0.18	0.10	0.12	0.04	0.35	0.19	0.93	0.28	0.18	0.14	0.47	0.13	0.11	0.13
CD3+CD4+/ CD3+CD8 ratio	3.65	2.32	2.36	3.60	2.19	1.22	1.13	1.04	1.89	2.00	2.26	1.48	6.50	2.13
HLA-DR+	0.18	0.08	0.22	0.12	0.18	0.11	0.34	0.11	0.20	0.12	0.39	0.16	0.16	0.60
Activated Lymphocytes	0.01	0.00	0.01	0.01	0.00	0.01	0.06	0.01	0.02	0.01	0.01	0.01	0.01	0.01
B Lymphocytes (CD 19+)	0.14	0.07	0.20	0.12	0.17	0.11	0.20	0.07	0.19	0.11	0.39	0.07	0.14	0.58
NK Lymphocytes (CD 16+)	0.05	0.09	0.2	0.08	0.35	0.50	0.58	0.30	0.19	0.25	0.04	0.07	0.11	0.06

**baseline, immediately before methylprednisolone pulse*

***four hours after methylprednisolone pulse*

Abbreviations: SLE = Systemic Lupus Erythematosus, MGN = membranous Glomerulonephritis, RPGN = Rapidly Progressive Glomerulonephritis.

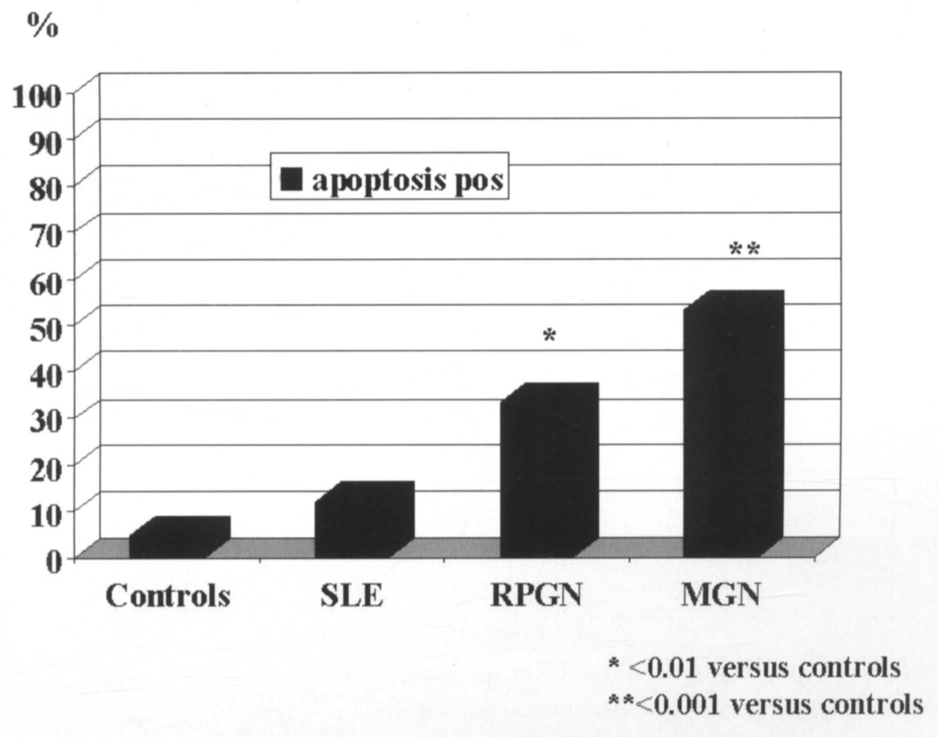

Fig. 1. *Frequency of positive apoptosis evaluated by DNA fragmentation in peripheral lymphocytes white cells in 22 Controls and in patients with different renal disease: 25 with Systemic LupusErythematosus (SLE), 6 with Rapidly Progressive Glomerulonephritis (RPGN), 19 primary Membranous Glomerulonephritis (MGN).*

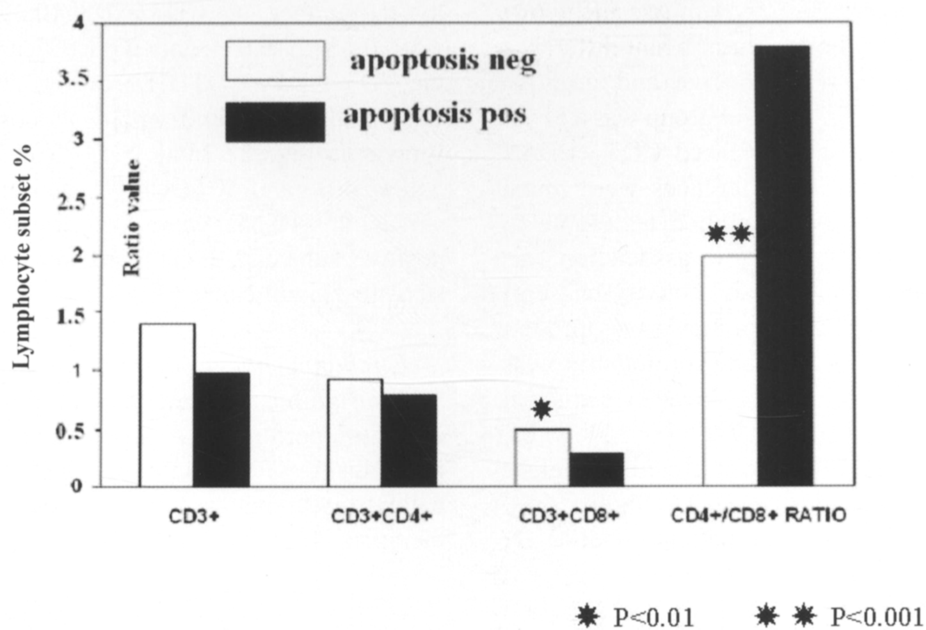

Fig. 2. *The overall difference between subjects with positive (n 16) and negative (n 63) baseline apoptosis (all groups, including controls) in CD3+, CD3+CD4+, CD3+CD8 and CD3+CD4 / CD3+CD8 ratio.*

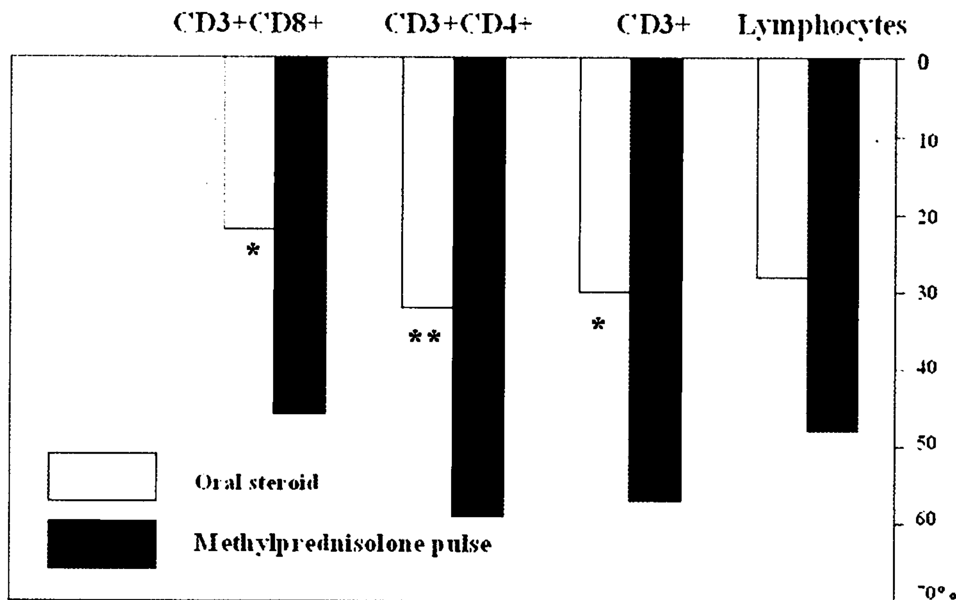

Fig. 3. *The effects of oral steroid therapy (total=23 patients, white bars) and intravenous methylprednisolone pulses (total=5 patients, black bars) in patients with SLE, MGN and RPGN patients: there was a sharper decrease in total lymphocytes, CD3+, CD3CD4+ and CD3CD8+ following methylprednisolone pulses.*

*** <0.01**
****<0.001**

two on mild activity phase), 2/6 of RPGN (33.3%, one Wegener and one Goodpasture syndrome, both on activity phase) and 10/19 of MGN (52.6%, five on remission, three on mild activity phase and two in acute phase) (Fig.1). The only significant difference between the subjects with positive and negative baseline apoptosis within each subgroup was a lower number of CD3+CD8, an increased CD3 +CD4 / CD3+CD8 ratio, while no differences were found in HLA-DR+ and activated lymphocytes (Table I). From a clinical point of view, no association was found with positive apoptosis and activity phase of SLE. In the case of MGN, apoptosis was present in patients in acute phase, and proteinuria was higher, though not reaching the level of statistical significance (2.2±3.1 versus 1.7 ±1.3 g/ 24 hours, NS), without difference in serum creatinine values (1.03±0.15 mg/dl versus 1.0 ±0.14 mg/dl, NS). Furthermore, all but one of the patients with MGN who showed positive apoptosis at baseline suffered from a relatively "young" disease, with a follow-up from renal biopsy <3 years. Within the subgroup of RPGN, apoptosis was both present and absent in 2 out of 3 categories of disease (Table I).

The overall difference between subjects with positive (n 16) and negative (n 63) baseline apoptosis (all groups, including controls) was the significantly lower number of CD3+CD8 (0.29±0.15 versus 0.50±0.15, $p<0.01$) and a significant increase in the CD3+CD4 / CD3+CD8 ratio (3.79±1.40 versus 1.98±0.24, $p<0.001$) in apoptosis positive-subjects, shown in Fig. 2. Total CD3+ (0.97±0.50 versus 1.40±0.40) and CD3CD4+ subsets (0.79±0.35 versus 0.92±0.25) were also lower in apoptosis positive subjects, thought not reaching a level of statistical significance (Fig. 2)

Circadian changes of apoptosis

No significant changes were found during the check-up performed 4 hours later in 10 healthy individuals, 5 RDT patients, 10 SLE and 10 MGN patients with inactive renal diseases and without therapies (Table II).

Changes in apoptosis following steroid therapy

The effects of oral steroid therapy in 10 SLE, 6 MGN and 6 RPGN patients are shown in Table III. There was a significant decrease in total lymphocytes

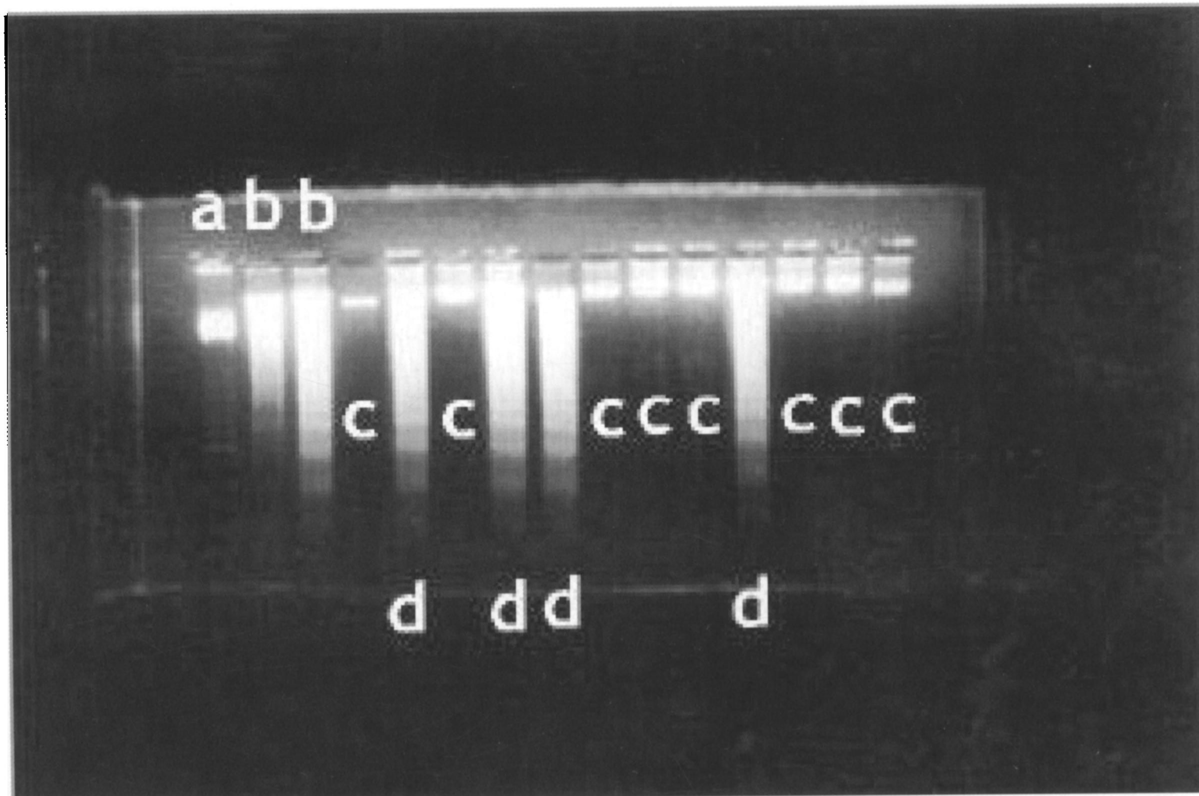

Fig. 4. *DNA electrophoresis showing positive samples with apoptosis indicated by DNA fragmentation, as compared to samples without DNA fragmentation, positive controls and marker of molecular weight; a) marker of molecular weight with bands between 100 and 300 pb; b) positive controls with typical DNA fragmentation of small molecular weight; c) negative samples without DNA fragmentation; d) positive samples with DNA fragmentation.*

(-28± 15%), CD3+ (-30±17%), CD3CD4+ (-32±15%) and CD3CD8+ (-22±20%) (Fig. 3. white bars), but baseline apoptosis did not change in any patient.

The effects of intravenous methylprednisolone pulses are shown in Table IV. A sharper decrease in lymphocyte total (-48±20%) and subset CD3+ (-57±20%), CD3CD4+ (-59±23%) and CD3CD8+ (-36±27%) was found (Fig. 3. black bars), while apoptosis turned from positive to negative in 2/2 MGN but not in 2 RPGN (the patients are the same as those studied during the phase of oral steroid therapy, as depicted in the previous table), and from negative to positive in 1/2 AIN and 1/1 SLE (Fig. 4).

DISCUSSION

Modulation of peripheral blood lymphocyte subsets during methylprednisolone pulse therapy is not a new discovery, and it is well-known that a more pronounced drop can be observed with methylprednisolone pulses than with continuous oral pulses, which has a direct correlation with the clinical efficacy of the treatment (13). Alteration of T-lymphocyte subpopulations in patients with renal diseases and systemic lupus has already been demonstrated, suggesting a role for alteration in T-helper subsets in the pathogenesis (14). What our study contributes to this setting is the first demonstration that a high percentage of patients with MGN have apoptosis in peripheral lymphocyte, and that methylprednisolone pulses turn this apoptosis from positive to negative.

A growing body of studies has been devoted to the role of apoptosis in renal physiology and during the course of progressive renal dysfunction (15-16). In particular, an altered balance between proliferation and apoptosis of mesangial and epithelial cells has been advocated in the pathogenesis of proliferative glomerulonephritis, as suggested from

both experimental and clinical settings (17-18). Furthermore, many studies concerning apoptosis and renal disease focused on SLE nephritis, dealing not only with the previously mentioned abnormalities of lymphocyte eventually linking the defects recorded in both T and B cells in lupus patients, but also with intra-renal apoptotic alterations involved in the pathogenesis of lupus nephritis, such as intense proliferation without consistent increment in apoptosis, eventually leading to an increased proliferation/apoptosis ratio (19-21). Moreover, derangement in intra-renal apoptosis has been demonstrated in IgA glomerulonephritis (22-23) diabetes nephropathy (24), polycystic kidney disease (25), Alport and Balkan nephropathies (26-27), acute renal failure from ischemic and toxic causes (28), hemolytic uremic syndrome (29), cyclosporine nephrotoxicity (30), obstructive nephropaties (31), amyloidosis (32), transplant nephropathy (33), focal segmental glomerulosclerosis (34), renal cancer (35) and different experimental models of nephritis such as acute puromycin aminonucleoside nephrosis (36), anti basal membrane (37) and nephrotoxic (38) nephritis.

However, no data have been published on apoptosis in MGN, except for one paper demonstrating apoptosis-associated cleavage products of DNA in the urine of patients with MGN (39). Our result is intriguing, as membranous GN is typical of GN in which cell proliferation, i.e. the process in which an abnormal apoptosis has been suspected to work at an increased level, is completely lacking. Understanding the meaning of this frequent apoptosis in MGN is beyond the scope of this study. We might just speculate that primary CD3+CD8+ expansion, Th2 oriented, (followed by increased apoptosis) could be responsible for overproduction of antibodies, eventually involved in that immunocomplexes glomerular deposition, which is the hallmark of MGN.

Further studies on larger samples are needed to confirm our results and to understand whether lymphocyte and renal cell abnormalities are consistent with this hypothesis. At present, it has only been demonstrated that patients with MGN have an overactive suppressor cell population or a numerical imbalance between CD4 and CD8 subsets with an increased ratio. Furthermore, it has been shown that an increased higher helper-inducer/suppressor-cytotoxic cell ratio before therapy may be a good prognostic index of proteinuria (40).

As for the results of steroid therapy on apoptosis, previous studies have already demonstrated apoptosis induction in human peripheral blood T lymphocytes by high-dose steroid therapy (41). What our study adds on this topic is the demonstration of a dual effect in patients with renal diseases, turning from positive to negative in two cases with MGN and from negative to positive one case with acute SLE nephritis and another case with AIN. The check-ups performed on a healthy population and patients with inactive renal diseases ruled out the possibility of circadian changes responsible for different results at 08 and at 12 am, that was previously demonstrated for lymphocyte subsets only from 08 and 21 (42). Therefore, changes in apoptosis from 08 to 12 may be assumed as due to therapy, that is methylprednisolone pulses.

The only other clinical setting in which the dual effect of methylprednisolone pulse on apoptosis has been reported is multiple sclerosis, where steroids may induce T-cell apoptosis in a CD95–independent, but caspase-dependent manner, or may protect cells from CD95-mediated apoptosis, which is however, also caspase dependent (43). In our case, as clinical benefits during the course of the disease were observed both in cases where apoptosis turned from positive to negative (MGN) and in cases in which apoptosis turned from negative to positive, we could speculate that methylprednisolone pulses were able to change deranged baseline patterns in opposite ways, eventually leading to a therapeutic effect by the normalisation of baseline opposite derangements in apoptosis. This dual effect is consistent with previous data from our group demonstrating that baseline lymphocyte subsets in patients with SLE nephritis were distributed very widely, with a CD3CD4+/CD3CD8+ ratio ranging from 0.26 to 2.7, and that correction after the first year of immunosuppressant therapy meant a normalization towards a ratio around a value of 1 in all cases: this meant an increase for cases with the lowest ratios, and a decrease for the cases with the highest ratios, as therapy was curative in opposite ways (44).

In conclusion, we have demonstrated that baseline apoptosis is frequent in a particular type of glomerulonephritis, that is membranous GN, and that methylprednisolone pulses have a dual effect

on peripheral lymphocyte apoptosis in patients with different renal diseases in acute phase, turning from positive to negative in two cases with MGN and from negative to positive in one case with SLE nephritis and in one case with ARF. The most important bias of our study is that the number of cases is small and that we did not specify in which lymphocyte subset apoptosis was present. Further studies are required to confirm our results and to verify our hypotheses.

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