

# Caspase 3 blocking avoids the expression of autoantigens triggered by apoptosis in neonatal Balb/c mice skin

*Il blocco della caspasi 3 inibisce l'espressione di autoantigeni indotti dall'apoptosi nella cute di topi Balb/c*

R. Ramírez-Sandoval<sup>1,2</sup>, M. Vázquez-del-Mercado<sup>3</sup>, A. Daneri-Navarro<sup>2</sup>, J.J. Bollain-y-Goytia<sup>1</sup>, E. Avalos-Diaz<sup>1</sup>, R. Herrera-Esparza<sup>1</sup>

<sup>1</sup>Departamento de Inmunología UBE y Área de Ciencias de la Salud.

Universidad Autónoma de Zacatecas, 98600, Guadalupe, Zacatecas, México;

<sup>2</sup>Programa de Doctorado en Ciencias Biomédicas, Departamento de Fisiología. Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, 44340 Guadalajara, Jalisco, México;

<sup>3</sup>Instituto de Investigación en Reumatología y del Sistema Músculo Esquelético Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, 44340 Guadalajara, Jalisco, México

## RIASSUNTO

**Obiettivo:** Valutare l'effetto terapeutico del blocco della caspasi-3 sull'espressione degli antigeni intracellulari indotti durante apoptosi.

**Materiali e metodi:** Lo studio è stato condotto in espianti di cute proveniente da topi Balb/c. I tessuti ottenuti dalle biopsie sono stati coltivati in DMEM. L'apoptosi e/o la necrosi sono state indotte mediante l'utilizzo di prodotti chimici. L'espressione degli antigeni Sm, RNP, Ro, La, dei corpi di Cajal e NuMa è stata valutata mediante immunostochimica o anticorpi monoclonali contro questi antigeni.

**Risultati:** Negli espianti di cute trattati con i prodotti chimici è stata osservata un'aumentata espressione degli antigeni intracellulari. L'inibitore DEVD-CMK della caspasi-3 ha indotto una diminuzione significativa dell'espressione degli antigeni nei tessuti trattati con i prodotti chimici. Dai risultati ottenuti possiamo concludere che gli inibitori delle caspasi sono in grado di modificare e diminuire gli autoantigeni associati alla morte cellulare.

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## ■ INTRODUCTION

Apoptosis is a physiologic process that maintains the cellular exchange; normally the cellular remains are cleared by phagocytosis without inflammation. In autoimmunity defects in apoptosis may occur, which play an important role in pathogenesis. During the lymphoid ontogeny the failure of the Fas pathway could decrease the negative selection process and such defect could allow the survival of autoimmune lymphocyte clones resulting in a central tolerance breaking down; in other instances the peripheral tolerance is disrupted by an insufficient clearance of apoptotic material, which is a potential risk for activating the pre-existent auto-reactive clones (1-3).

Experimental evidences indicate that antigens from apoptotic cells are better targeted by autoantibodies (4-6). Physical factors like the UV irradiation or caloric stress may increase the expression of autoantigens. Experimentally the UV irradiated skin is better targeted by anti-Ro antibodies. This phenomenon is observed in the skin of patients with sub-acute cutaneous lupus erythematosus (SCLE). In those patients the autoantigen availability increases after sun exposure and such factor may induce *in situ* formation of Ro/anti-Ro immune complex (7). The aim of this study was to assess the effect of caspase 3 blocking in the expression of Sm, La, Ro, RNP, Cajal bodies and NuMa antigens associated to apoptotic or necrotic keratinocytes.

Corresponding author:  
Rafael Herrera-Esparza, MD  
Chepinque 306, Col.  
Lomas de la Soledad  
Zacatecas 98040 México  
E-mail: herrerar@uaz.edu.mx  
rafael.herreraesparza@gmail.com

## ■ MATERIAL AND METHODS

### *Skin biopsies*

Neonatal Balb/c mice were maintained on ice bath 30 minutes before biopsy procedure. Skin biopsies were removed by 3 mm punch, tissues were rinsed in Hank's solution and cultured in DMEM medium (Dulbecco's modified Eagle Medium) supplemented with 10% fetal calf serum (Gibco, BRL Gaithersburg, MD).

### *Antibodies*

Anti-Ro (sc-19681 Santa Cruz, Biotechnology, Inc.), anti-La was obtained from a patient with Sjögren disease (8), anti-Sm antibody was obtained from a systemic lupus erythematosus (SLE) patient which met the ACR criteria for disease classification (9), anti-RNP serum was obtained from a patient with mixed connective tissue disease (10), anti-NUMA was obtained from a patient with scleroderma (11) and anti-Cajal body serum was obtained from a SLE patient (12). Antibodies were tested by indirect immunofluorescence on HEp-2 cells. Affinity purified autoantibodies were obtained as previously described by elution with 0.2 M glycine HCl, pH 2.8, then neutralized with 1 M Tris, pH 8.6, and concentrated by ultrafiltration (13).

### *Chemical induction of apoptosis and necrosis*

Skin biopsies were incubated in polystyrene dishes at 37°C in 5% CO<sub>2</sub>. Biopsies were grouped in number of five, they were incubated in Petri dishes during 12 h in DMEM; skin fragments of each group was cultured as follows:

- A) Control group without chemicals.
- B) 20 µM of camptothecin (CPT).
- C) 20 µM of cycloheximide (CHX).
- D) 0.2% H<sub>2</sub>O<sub>2</sub>.
- E) 5 mM of HgCl<sub>2</sub>.

A duplicate of each group was previously incubated for 2 h with the synthetic peptide DEVD-CMK (Calbiochem) which is a caspase 3 inhibitor; the peptide was dissolved in DMSO and adjusted to 20 mM in PBS, and then incubated with tissues, followed by incubation with aforementioned chemi-

cals (7). A course time experiment was carried out at 12, 24, 48 and 72 h; in this assay 5 biopsies by each experimental condition were included. After incubation all skin biopsies were fixed in 10% neutral formalin, then embedded in paraffin and used for immunohistology.

During this trial the guidelines for ethical conduct in the care and use of animals developed by American Psychological Association (APA) was followed (<http://www.apa.org/science/anguide.html>).

### *Immunohistochemistry*

Slides containing 4 µm sections of skin were dewaxed and permeabilized with 0.01% Triton X-100/PBS, then were washed three times with PBS; endogenous peroxidase was blocked with horse serum inactivated at 56°C, after washings, the tissues were incubated 1 hour with the monoclonal antibody or human affinity purified autoantibodies diluted 1:100 in 10% fetal calf serum/PBS; after washings with PBS, a new 1 h incubation with rabbit anti-mouse or goat anti-human IgG labeled with peroxidase was performed, after washings color reaction was induced by 3,3'-diaminobenzidine-0.06% H<sub>2</sub>O<sub>2</sub> (Sigma, San Louis, MO), finally the reaction was stopped with 2N sulphuric acid.

Assays were made by duplicate. The color intensity of immunohistochemistry reaction was measured using a Bio-Rad Quality One analyzer and expressed as the sum of pixels intensity.

### *TdT-mediated dUTP nick end labeling (TUNEL)*

The assay was done according to the manufacturer's instructions (Roche Molecular Biochemicals, Penzberg, Germany).

Nuclear stripping was performed immersing the slides in 10 mM Tris-HCl pH 8.0, followed by 15 min in 20 mg/ml proteinase K. The elongation of DNA fragments was done by incubation with reaction mixture (DDW, 10X TdT buffer (30 mM Tris base, 140 mM sodium cacodylate, pH 7.2, 1 mM cobalt chloride, 1 mM DTT; 10% of the final volume), fluorescein-11-dUTP (0.5 mg dissolved in 1 ml of 10 mM Tris-HCl,

pH 7.0), and TdT enzyme 0.3 units/ml). Reaction was terminated by adding 300 mM NaCl, 30 mM sodium citrate, pH 8.0. Slides were washed in PBS.

To differentiate the true green tag of apoptotic cells from the background incorporation, tissues were counterstained with 2% propidium iodide.

By this procedure the non-apoptotic nuclei developed a red stain.

The slides were washed, mounted, and examined using a confocal scanning microscope LSM (Axiovert 200M, Carl Zeiss, Göttingen, Germany).

Combination of fluorescein filters with excitations of 450–490 nm and rodamin filters with emissions of 515–565 nm were used. Objectives were LCI “Plan-Neofluar” and image processing was done with a Zeiss LSM Image examiner. The rate of apoptotic cells by TUNEL was calculated taking into account the percent of cells exhibiting green tags in 100 fields.

A positive control was included in all experiments (skin biopsies digested with nucleases). Slides were evaluated by three different observers in a blind manner.

### Statistics

Differences between samples were evaluated using non parametric statistics (ANOVA and unpaired t test) using the PRISMA program.

## ■ RESULTS

### *Skin explants cultures*

The culture of Balb/c mouse skin was successful; and cell viability determined by TUNEL was ~99% in skin cultured without chemicals, and was taken as base control.

### *Induction of apoptosis or necrosis*

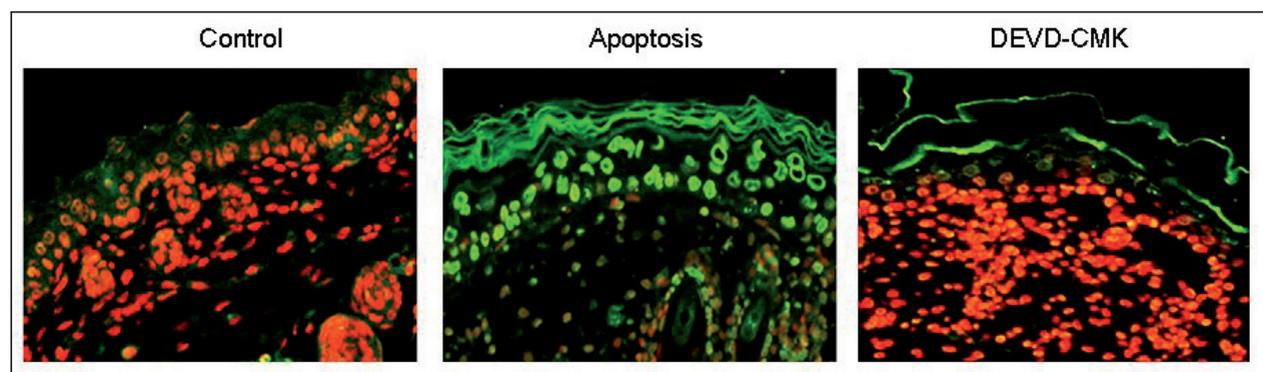
With the notion that camptothecin and cycloheximide induce apoptosis and the hydrogen peroxide and mercury chloride induce secondary necrosis, we used this approach to trigger cell death, which was monitored by TUNEL.

High cell death index was observed in skin explants cultured with chemicals and was significantly different from controls (Tab. I, Fig. 1).

**Table I** - Cell death chemically induced measured by TUNEL.

Control	CPT	CHX	HgCl <sub>2</sub>	H <sub>2</sub> O <sub>2</sub>
0.9000±1.595	75.60±17.46	88.40±11.07	86.90±10.16	66.30±20.36
vs	P<0.0001	P<0.0001	P<0.0001	P<0.0001

ANOVA

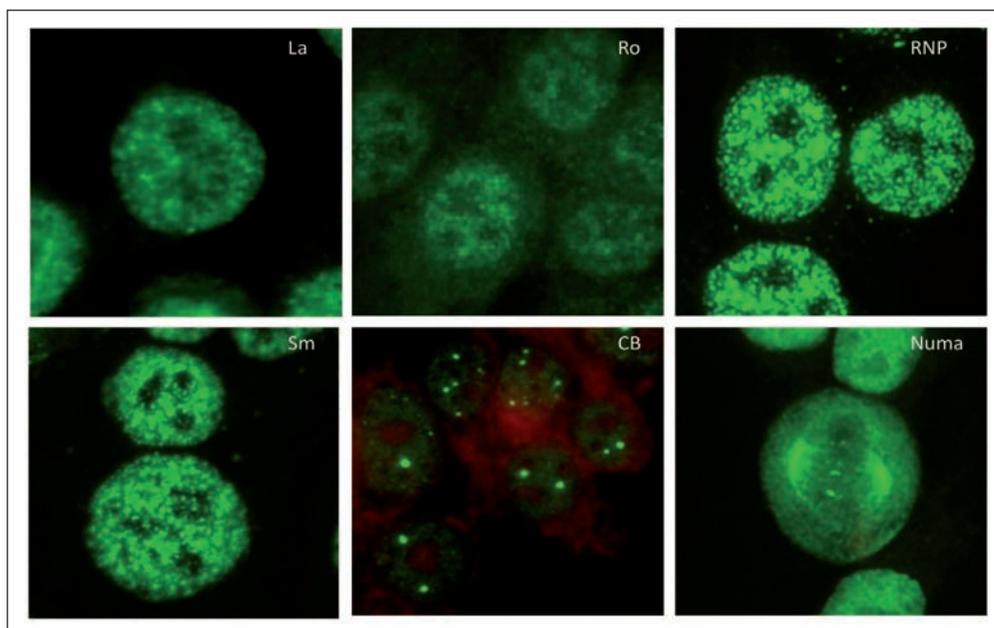


**Figure 1** - Cell death induced by camptothecin. On the left a representative TUNEL assay by fluorescence; the green tags indicated apoptotic cells, meanwhile the red tags corresponds to the propidium iodide incorporation by the non-apoptotic cells. Negative control corresponds to a skin section incubated in DMEM medium without chemicals. In the center, the skin shows apoptotic keratinocytes after incubation with camptothecin. On the right the caspase 3 inhibitor DEVD-CMK impedes apoptosis.

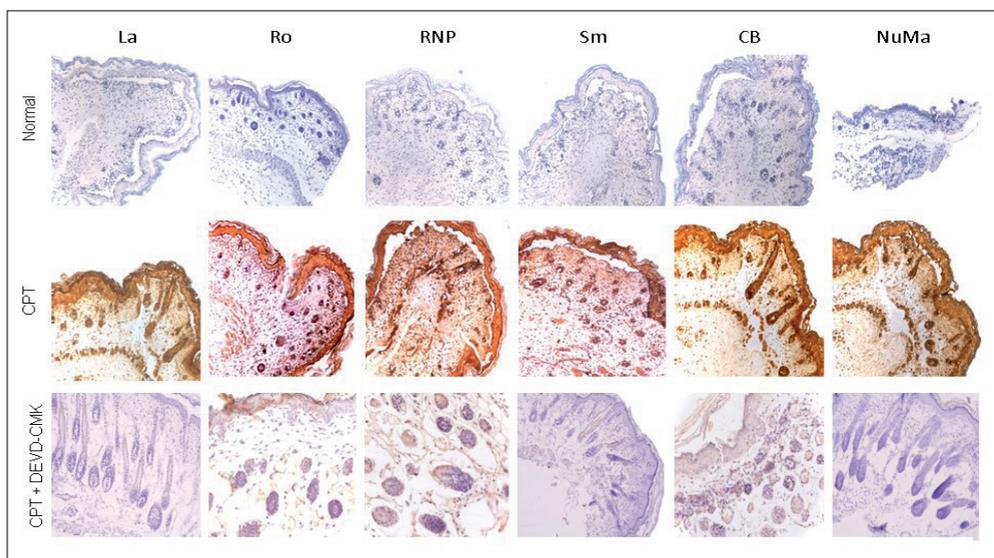
***The cell death releases intracellular antigens of the skin***

The antibody reactivity against La, Ro, RNP, Sm, Cajal bodies (CB) and NuMA antigens was firstly tested on HEp-2 cells to obtain the fluorescent pattern (Fig. 2), and

then tested on skin explants cultured without chemicals resulting in faint staining in normal skin, nevertheless it significantly increased in apoptotic and necrotic tissues (Fig. 3). Antigens reach the highest expression 48 h after incubation with chemicals,



**Figure 2** - Fluorescent patterns of antibodies used in the present study and tested by indirect immunofluorescence on HEp-2 cells.



**Figure 3** - Antibodies reactivity in mouse skin tissues. On the superior panel a representative immunohistochemistry of normal skin probed with different antibodies. In the middle the skin was treated with camptothecin, shows that apoptosis increases the antigenic expression. In the inferior panel, the caspase 3 inhibitor effect avoids the expression of different autoantigens.

**Table II** - Antigen expression by immunohistochemistry under apoptosis and necrosis chemically induced.

Condition	Control	12 h	24 h	48 h	72 h	p Value*
La-CPT	72.90±16.68	3383±2081	5654±2373	10940±6283	7063±2812.	<0.0001
La-CHX	41.30±13.38	13440±404	8075±5410	11760±3707	6849±2423	<0.0001
La-HgCl <sub>2</sub>	58.40±15.57	4960±2920	8638±2590	10030±3012	5636±2180	<0.0001
La-H <sub>2</sub> O <sub>2</sub>	57.40±10.33	4593±2292	3713±1048	7758±2274	5148±1311	<0.0001
Ro-CPT	51.00±14.49	4248±1340	9917±2694	14270±3112	8810±832.4	<0.0001
Ro-CHX	42.00±14.14	6050±1161	11170±2012	8854±2890	7059±1172	<0.0001
Ro-HgCl <sub>2</sub>	42.60±16.36	6901±803.	6341±1034	9538±1972	6871±2832	<0.0001
Ro-H <sub>2</sub> O <sub>2</sub>	70.22±26.47	7631±1712	13590±2128	12960±2815	6634±954.1	<0.0001
RNP-CPT	41.40±8.695	8260±1544	7716±1823	11600±2860	6007±2177	<0.0001
RNP-CHX	28.00±7.888	3892±9696	6359±1417	6415±2003	5495±1544	<0.0001
RNP-HgCl <sub>2</sub>	55.20±12.76	5361±1386	8143±2934	6350±2035	4735±1576	<0.0001
RNP-H <sub>2</sub> O <sub>2</sub>	29.00±5.164	5803±2363	6248±1897	3371±513.5	6038±469.1	<0.0001
Sm-CPT	45.50±11.07	5941±2477	4077±2163	4572±1337	4998±1302	<0.0001
Sm-CHX	41.30±11.64	4376±1382	4101±1063	3977±1037	4916±2558	<0.0001
Sm-HgCl <sub>2</sub>	26.20±10.68	1268±4010	2922±924.1	1177±372.2	2605±823.8	<0.0001
Sm-H <sub>2</sub> O <sub>2</sub>	24.60±11.11	4119±1370	3868±893.8	4576±1071	7210±1566	<0.0001
CB-CPT	57.60±11.81	6701±1949	4056±1734	8098±1493	8556±1880	<0.0001
CB-CHX	32.00±11.35	3359±9279	6682±2453	5371±802.4	6897±3010	<0.0001
CB-HgCl <sub>2</sub>	50.20±9.773	6123±1692	5397±1188	9003±2531	2588±816.2	<0.0001
CB-H <sub>2</sub> O <sub>2</sub>	35.50±7.976	6099±1729	6697±2184	4623±1397	4260±1091	<0.0001
NuMa-CPT	18.60±5.621	5091±1680	5014±1737	6619±3125	7157±2685	<0.0001
NuMa-CHX	35.50±6.852	3733±1965	6055±1311	10090±3706	9089±3760	<0.0001
NuMa-HgCl <sub>2</sub>	61.60±15.41	8199±2506	8199±2506	4051±1931	6580±1683	<0.0001
NuMa-H <sub>2</sub> O <sub>2</sub>	43.00±8.563	3789±1106	6627±1868	7569±2004	7284±1778	<0.0001

\*ANOVA

with exception of the La autoantigen that display a biphasic expression at 12 h and 48 h. The major expression of autontigens was obtained with camptothecin and cycloheximide (Tab. II, Fig. 4). Control serum from a healthy person resulted negative in all assays.

### ***The caspase 3 blocking decreases autoantigens expression***

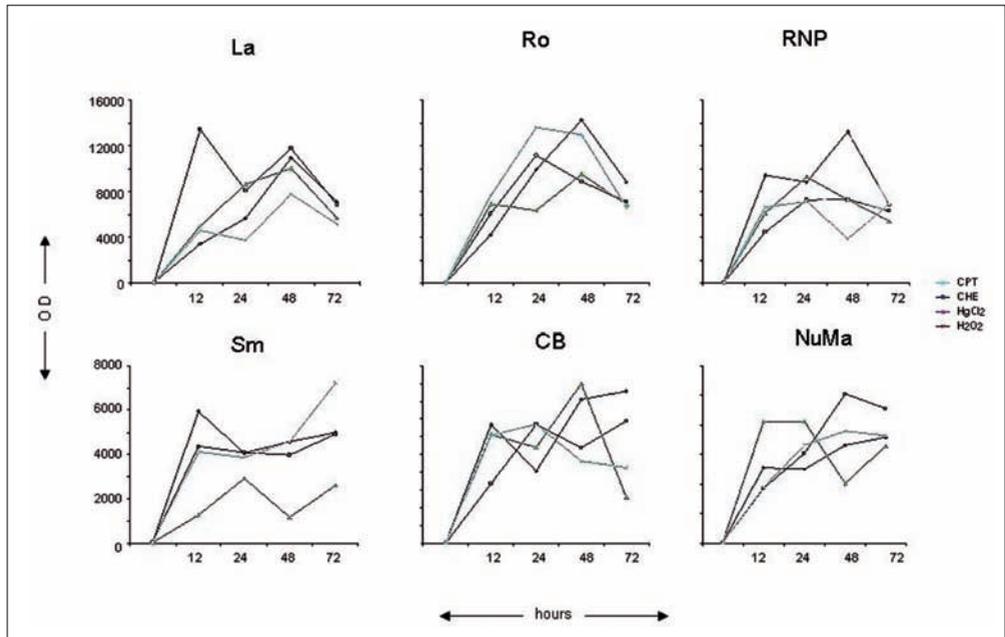
With the notion that apoptosis increases the expression of autoantigens, we assumed that the caspase 3 blocking would decrease the antigenic expression. The major finding of this experiment was that DEVD-CMK decreases the expression of intracellular antigens after 12 h incubation; therefore

tissues treated with this synthetic peptide, exhibited similar reactivity to autoantibodies than the control tissues (Fig. 5, Tab. III).

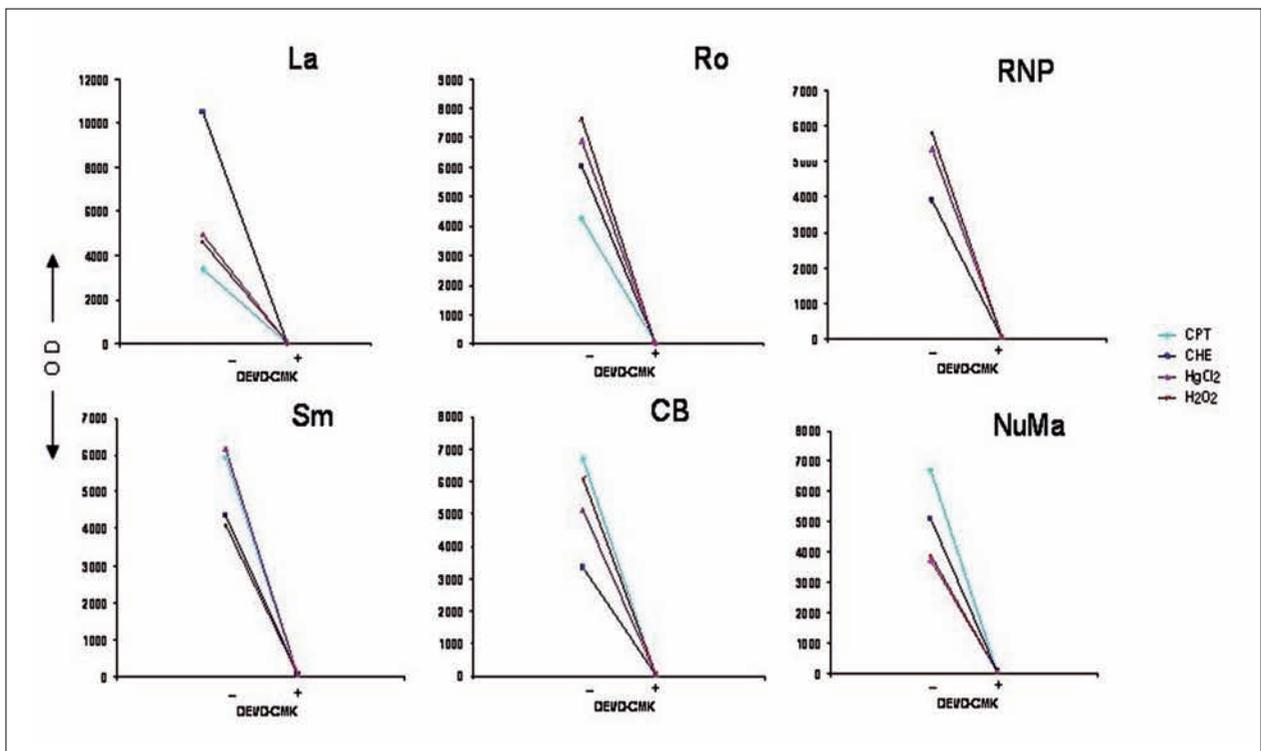
## ■ DISCUSSION

The present investigation addresses the question whether the caspase 3 blocking could decrease the autoantigen expression associated to cell death. The main results are the following:

- 1) Skin explants cultured and treated with pro-apoptotic/necrotic chemicals induce cell death *in vitro*;
- 2) Cell death index determined by TUNEL was significantly increased in skin ex-



**Figure 4** - Fate of autoantigens expression under apoptosis and or necrosis chemically induced, in a course time manner. CPT = camptothecin. CHX= cycloheximide. HgCl<sub>2</sub> = mercury chloride. H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide. The graph represents a semi quantitative measure of the color intensity in pixels. O.D = optical density.



**Figure 5** - Autoantigens expression is prevented by preincubation with the caspase 3 inhibitor DEVD-CMK, impedes apoptosis at 12 hours, and avoids the autoantigen release.

**Table III** - Effect of caspase-3 blocking in autoantigen expression.

Condition	Basal	12 h	12 h+DEVD-CMK	p value
La-CPT	72.90±16.68	3383±2081	51.50±13.34	<0.0001
La-CHX	31.73±50.87	10550±16340	55.50±11.41	<0.0001
La-HgCl <sub>2</sub>	58.40±15.57	4960±2920	53.10±11.90	<0.0001
La-H <sub>2</sub> O <sub>2</sub>	51.85±20.78	4593±2292	57.50±14.58	<0.0001
Ro-CPT	51.00±14.49	4248±1340	59.00±12.65	<0.0001
Ro-CHX	42.00±14.14	6050±1161	56.50±17.96	<0.0001
Ro-HgCl <sub>2</sub>	42.60±16.36	6901±803.2	49.50±12.12	<0.0001
Ro-H <sub>2</sub> O <sub>2</sub>	70.22±26.47	7631±1712	50.56±13.57	<0.0001
RNP-CPT	55.20±12.76	5361±1386	50.00±15.46	<0.0001
RNP-CHX	28.00±7.888	3892±969.6	32.40±13.98	<0.0001
RNP-HgCl <sub>2</sub>	55.20±12.76	5361±1386	56.00±6.992	<0.0001
RNP-H <sub>2</sub> O <sub>2</sub>	29.00±5.164	5803±2363	51.50±7.091	<0.0001
Sm-CPT	45.50±11.07	5941±2477	51.50±12.70	<0.0001
Sm-CHX	41.30±11.64	4376±1382	52.00±10.59	<0.0001
Sm-HgCl <sub>2</sub>	26.20±10.68	6164±1268	56.50±20.15	<0.0001
Sm-H <sub>2</sub> O <sub>2</sub>	24.60±11.11	4119±1370	51.00±17.76	<0.0001
CB-CPT	57.60±11.81	6701±1949	50.50±13.01	<0.0001
CB-CHX	32.00±11.35	3359±927.9	55.50±19.07	<0.0001
CB-HgCl <sub>2</sub>	50.20±9.7736	6123±1692	53.50±12.26	<0.0001
CB-H <sub>2</sub> O <sub>2</sub>	35.50±7.976	6099±1729	63.50±9.733	<0.0001
NuMa-CPT	18.60±5.621	5091±1680	60.00±22.73	<0.0001
NuMa-CHX	35.50±6.852	3733±1965	56.50±11.07	<0.0001
NuMa-HgCl <sub>2</sub>	61.60±15.41	4439±918.5	55.00±14.72	<0.0001
NuMa-H <sub>2</sub> O <sub>2</sub>	43.89±8.580	3900±1113	60.50±11.89	<0.0001

Differences between 12 h vs 12 h+DEVD-CMK by t Test.

plants treated with pro-apoptotic chemicals;

- 3) Apoptotic or secondary necrotic process enhances the expression of intracellular antigens on the surface of apoptotic membranes, therefore apoptotic cells became easy targets for autoantibodies;
- 4) Antigen expression is decreased by caspase 3 inhibitors.

Physical factors like UV irradiation and caloric stress are associated to lupus flare-ups, mechanisms involved at molecular level are still under investigation, for instance UV irradiation induces apoptosis, which together with a defective clearance of apoptotic material may induce infiltration of macrophages and recruitment of plasmacytoid dendritic cells (DCs). The interaction be-

tween DCs and CD4 cells produce IFN- $\gamma$ , TNF, IL-1 cytokines and induce cytotoxic CD8-mediated mechanisms, B cell activation with autoantibody production and immune complex formation and all together produce tissue damage (14-17). A complex network of IFN-associated cytokines, chemokines (MxA, CXCL10) and adhesion molecules orchestrates and promotes skin damage in lupus (18). With the notion that apoptosis is important in lupus, we infer that inhibition of caspases pathway could decrease the cutaneous expression of autoantigens dependent of apoptosis; this issue is of clinical importance because it might represent a promising associated therapy in lupus relapses. In this context we used camptothecin and other pro-apoptotic chemical agents,

because under camptothecin-induced apoptosis the activation of effectors caspases -7 and -3, and poly(ADP-ribose) polymerase (PARP) cleavage, occurs as early events of apoptosis which precede the release of cytochrome c and apoptotic inducing factor, as well as the activation of caspases 2, 8, 9 and 12 (19). Theoretically the caspase 3 inhibition by means of a synthetic peptide such as DEVD-CMK inhibit the cell death and might decrease the autoantigen release, with this concept the caspase pathway blocking was used to improve lupus nephritis in IFN- $\gamma$ -transgenic mice (CBA x C57/BL10 F1s); therefore the administration of Carbobenzoxy-Valyl-Alanyl-Aspartyl-( $\beta$ -o-methyl)-Fluoromethylketone a caspase 3 inhibitor delay the nephritis development in experimental animals (20, 21).

We focus our pharmacologic intervention to block caspase 3, because is a essential key in apoptosis, therefore the activation of the zymogen procaspase-3 to caspase-3 leads apoptosis, this "executioner enzyme" catalyzes the hydrolysis of multiple protein substrates within the cell and enhances the auto antigens expression. Innovative therapeutic approaches are currently being used to treat humans or experimental models of cancer, degenerative and autoimmune diseases, among these therapies are the recombinant biomolecules, antisense oligonucleotides, gene therapy and combinatorial chemistry to target specific apoptotic

mediators; such advances allowed the specific inhibition of caspases pathway in different phases, therefore this pharmacologic intervention permit to modify molecular mechanisms for autoimmune diseases like the SLE (22). Caspase 3 play another role in SLE, because T cells of these patients express low levels of CD3 $\zeta$ -chain, this is probably caused by an increased caspase 3 expression and activity, which cleaves the CD3 $\zeta$ -chain, the proteolysis result in an aberrant TCR/CD3-mediated signaling in SLE T cells, furthermore treatment of T cells with caspase 3 inhibitor Z-Asp-Glu-Val-Asp-FMK normalize T cell function by preventing the excessive digestion of CD3 $\zeta$ -chain, this induce restoration of the defective IL-2 production.

This and other experimental data support the notion that molecules that modify apoptosis might be considered in a future for treatment of systemic autoimmune disease including lupus (23). To summarize different experimental approaches indicate that the caspase intervention at molecular level constitute a promising research area, such strategy in theory can modify the lupus flare-ups.

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#### **SUMMARY**

**Objective:** *To assess the effect of caspase 3 inhibition, in the expression of intracellular antigens induced by apoptosis.*

**Material and methods:** *Skin explants of neonatal Balb/c mice were used to assess the autoantigen expression. Skin was obtained by punch biopsies, tissues were cultured in DMEM; cell death was induced by chemicals and assessed by TUNEL. The expression of La, Ro, Sm, RNP, Cajal Bodies and NuMa antigens were monitored by immunohistochemistry using autoantibodies or monoclonal antibodies against these antigens.*

**Results:** *Chemicals used to induce cell death, successfully produced apoptosis or necrosis in more than 60% of keratinocytes, and viability was significantly decreased when it was compared with those in controls. An increased expression of all skin intracellular antigens in skin biopsies treated with chemicals, major antigenic expression was detected with anti-La and anti-Ro antibodies. The caspase 3 inhibitor DEVD-CMK significantly decreased the expression of antigens induced by chemicals.*

**Conclusion:** *By this result we can infer that caspase inhibitors modify apoptosis and decrease the autoantigens associated to cell death.*

**Parole chiave:** Apoptosi, auto antigeni, inibitori della caspasi-3.

**Key words:** Apoptosis, autoantigens, caspase-3 inhibitor.

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