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LAVORO ORIGINALE

Ro60 and La ribonucleoproteins become self-aggregated by cell stress

Lo stress cellulare induce l'autoaggregazione delle ribonucleoproteine Ro60 e La

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RIASSUNTO

Gli antigeni Ro e La sono d'interesse clinico nel lupus subacuto cutaneo in quanto le lesione cutanee che appaiono dopo esposizione solare inducono la traslocazione delle ribonucleoproteine intracellulari Ro e La e innescano la produzione di autoanticorpi. L'obiettivo del nostro studio è determianare se lo stess cellulare è in grado di modificare le caratteristiche molecolari e la distribuzione delle ribonucleoproteine Ro e La. A tale scopo cellule HEp-2 sono state sottoposte a stress cellulare utilizzando calore e radazioni UV, e l'espressione di Ro e La è stata valutata mediante immunofluorescenza, western blot e inmunoprecipitacione utilizzando anticorpi monoclonali anti-Ro/La o anti-HSP70 legati a sepharosa-4B. I risultati confermano che in condizioni di stress cellulare Ro e La, presenti nel nucleo, si ridistribuiscono nel citoplasma e nucleoplasma. Inoltre, lo stress induce l'autoaggregazione delle ribonucleoproteine, come dimostrato dal western blot. Le proteine interagiscono con il citoscheletro via HSP70.
In conclusione, in condizioni di stress cellulare le proteine Ro e La si ridistribuiscono all'interno del compartimento nucleo-citoplasmatico. Tale ridistribuzione è accompagnata da autoaggregazione e associazione con HSP70. Lo stress cellulare rappresenta quindi un fattore importante per la ridistribuzione antigenica.

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INTRODUCTION

Ro is a cellular particle composed by three ribonucleoproteins of 60, 54 and 52 kDa (1-3). Ro60 forms a complex with one of the 1-5 hYRNAs (4). Antigenic properties of Ro were described by Clark in 1969 using autoimmune sera (5), and it is broadly accepted that Ro is recognized by autoantibodies from patients with systemic lupus erythematosus (SLE), subacute cutaneous lupus erythematosus (SCLE), Sjögren's syndrome (SS) and neonatal lupus (6). Complexes of Ro are involved in the transcription quality control of 5S rRNA, Ro60 bind the 5S rRNA inefficient transcribed to be eliminated (7, 8). Ro is expressed broadly in nucleus and cytoplasm of eukaryotic cells (9-11). Anti-Ro antibody is of clinical interest because is associated to SCLE. Apparently Ro is released by keratinocytes exposed to UV irradiation, which presumably trigger the cutaneous lesions by local anti-Ro/La antibodies deposition, that in turn activate a cytotoxic mechanism dependent of antibodies (6). Recently has been disclosed that Ro52 is a RING-dependent E3 ligase involved in ubiquitination, and its increased expression in patients may be directly involved in reducing cellular proliferation and increasing apoptotic cell death observed in Sjögren's syndrome and SLE, this is important because a enhanced expression of Ro52 in tissues, result in an increase of apoptoic remains in tissues (12).

La antigen is a 48 kDa ribonucleoprotein that plays a role in transcription as termination factor (13). The activity of La antigen depends on phosphorylation and de-phosphorylation cycles (14); this process also affects its antigenicity (15, 16). La autoantibody is of clinical interest because of its association with the Sjögren syndrome and SCLE (6). Sera with anti-La specificity generally possess

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anti-Ro antibodies; this suggests that Ro and La antigens are clustered (17, 18).
In SCLE, solar irradiation constitutes the major stressor of the skin, because it contains both UV waves and caloric stress; in SCLE the relapses of skin lesions are frequently associated with sun exposure, which probably enhances the antigen expression that is followed by auto-antibody production. The exposure to those stressors triggers a physiological response and the transcription of heat shock proteins (HSP); being the most important HSP70, which play a role in degradation, refurbishing and reactivation of damaged proteins.
Present studies address the issue whether the stress affects the expression, distribution and possible conformational changes of Ro60 and La antigens, and its possible association with the cytoskeleton. Since HSP70 is constitutive and ubiquitously protein distributed in cell compartments (19), HSP70 was included as monitor of cell stress, during this study its possible relationship with Ro, La and cytoskeleton proteins was assessed.

MATERIAL AND METHODS

Cell culture
HEp-2 cells (ATTC) were grown in DMEM media (Sigma St. Louis, Mo.) with penicillin (100 U/ml), streptomycin (100 mg/ml), insulin (0.08 U/ml), and 10% fetal bovine serum (Gibco BRL, Grand Island, N.Y) at 37.5°C, in a 5% CO_2 atmosphere of. Cells growing in logarithmic phase were harvested by means of trypsin EDTA and plated at confluence for experiments with stressors ($5x10^6$/ml).

Cell stressors
Cultures were irradiated at 366 nm using a UV-A lamp (Black-Ray lamp UVL-56), archiving a dose of 5-30 mJ/cm^2 (20). Control cells were cultured and manipulated without irradiation. Caloric stress was done by raising the temperature of cell cultures from 37°C to 40°C, 42°C and 44°C respectively, for a duration of 3 hours.

Protein extraction
Stressed and non-stressed cells were harvested as described and spun at 1200 rpm for 10 minutes. The cellular pellets were resuspended in denaturing lysis buffer (1% SDS, 0.25 M EDTA, 1 mM Tris, pH 7.5, and 1 mM PMSF; adjusted to 1 ml H2O). Soluble antigens were extracted by sonication: 5 pulses of 30 seconds at 50%. The lysates were centrifuged at 12000 rpm/10 minutes and the supernatants were immediately used for analysis. The protein concentration was measured at 280 nm. The insoluble fractions were extracted by 30 minutes incubation with a urea buffer [1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM PMSF, in 10 mM Tris-HCl, and pH 7.6 with 8 M urea].

ELISA test
Stressed and non-stressed HEp-2 cell extracts were used for coating the polystyrene plates, after washings with PBS; the uncoated sites were blocked 1 hour with 3% BFS-PBS. Following washings, the coated plates were incubated overnight with anti-Ro; anti-La or anti-HSP70 antibodies diluted 1:3000 in 3% BFS-PBS. Plates were further washed and bound antibodies were tagged with goat anti-mouse IgG HRP-labeled. Color was induced by 2, 2 diaminobenzydine and 0.06% H_2O_2. The reaction was stopped with 2 N sulphuric acid; optical density was determined at 490 nm in a Bio-Rad ELISA reader. All assays were made in triplicate (21). Data was processed by student t-Test (Prisma program); $p < 0.005$ was considered statistically significant.

SDS-PAGE and Western blot
Cellular extracts were submitted to 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as described by Laemmli and Favre (22); non-reducing conditions were also applied in the absence of bismercaptoethanol or DTT in the sample buffer. The unstained gels were blotted onto nitro-cellulose membranes using the procedure described by Towbin et al. (23). Non-specific active sites were blocked with 1% non-fat milk. Immunorreactive bands were identified with a 1:1000 dilution of monoclonal anti HSP70, anti-Ro or anti-La antibodies. 1:1000 in phosphate saline buffer (PBS). Bound antibodies were detected with rabbit anti-mouse IgG-HRPC (Sigma. St Louis, MO). Immunoreactive bands were visualized with an ECL chemiluminescent kit (Amersham Pharmacia Biotech. Piscataway, NJ). The bands were recorded using an image analysis system. The molecular weights at the immunoreactive bands could be determined by comparison with standards of known molecular mass (BioRad, Richmond. CA).

Crossed-Immunoprecipitation
Cell extracts were immunoprecipitated with anti-Ro, anti-La and anti-HSP70, anti-Tubulin, anti

Actin or anti-Cytokeratin antibodies linked to CN-Br activated 4B Shepharose (Sigma, St Louis Mo) and dissolved in 50 mM tris, pH 7.0. Cell extracts and immunoadsorbents were mixed, incubated and rocked at room temperature overnight. After extensive washings; immunoprecipitates were eluted with 0.1 M glycine at pH 3.0, followed by neutralization with 1 M Tris-base, pH 9.0; and characterized by SDS-PAGE, were blotted and tagged with anti-Ro, anti-La or anti-HSP70, or with anti actin, tubulin and cytokeratin, or vice versa, immunoreactive bands disclosed by autoradiography as previously described.

Double fluorescent labeling

Ro, La and HSP70 co-localization with cytoskeleton components was defined by a double labeling. The first incubation with anti-Ro, anti-La or anti-HSP70 was followed by 1 hr incubation with FITC labeled secondary antibody (sheep anti-mouse; Cappel, West Chester, PA). After extensive washings, a third incubation with anti-actin, b-tubulin and cytokeratin was carried out, followed by a red tagging with RITC-goat anti-mouse (Sigma). Finally, the slides were washed, mounted and examined under a confocal scanning microscope LSM (Axiovert 200M, Carl Zeiss, Göttingen Germany). Fluorescein and rodamine filter combinations with excitations of 450-490 nm and emissions of 515-565 nm were used respectively, objectives were LCI "Plan-Neofluar" and the image processing was done by a Zeiss LSM Image examiner.

Antibodies

The following monoclonal antibodies were used: anti-Ro60 monoclonal antibody which recognized a single band at 60 kDa level and anti-La monoclonal antibody recognizing a single 48 kDa band (24); these monoclonals were generously donated to our laboratory by R.T. Smeenk from the CLB (Amsterdam), also were used anti-HSP70 monoclonal antibody (H-5147) anti-actin (A-4700), anti-b-tubulin (T-5168) and anti-cytokeratins H-4840) which were obtained commercially (Sigma, St Louis MO).

RESULTS

Stress enhances Ro and La expression

HSP70 was used as cell stress monitor; and its expression was significantly superior in extracts of stressed cells as demonstrated the ELISA assays. According to our previous findings Ro and La expression were increased in extracts from cells UV irradiated or submitted to caloric stress (Tab. I).

Stress induces Ro and La self-aggregation

We were able to demonstrate by *Western blot* an increase of the Ro and La intensity bands of stressed cell extracts. Interestingly, the extracts of the soluble fraction exhibited two additional bands of Ro. Meanwhile La display additional bands mainly in the insoluble fraction of heated cells. To elucidate if bands corresponded to self-aggregate or belonged to degradation protein fragments, the cell extracts containing the possible aggregates of Ro and La, were additionally electrophoresed under reducing conditions. Interestingly, the bis-mercaptoethanol and/or DTT induced Ro and La disaggregating and produced unique bands; this result confirms that the dimmers and trimmers of Ro and La induced by stress correspond to self aggregates (Fig. 1).

Ro and La co-localize with the cytoskeleton

The presence of Ro and La proteins in the insoluble fraction of stressed cells, suggested some kind of association between these ribonucleoproteins and the cytoskeleton, to confirm such association a double fluorescence assay tagging the Ro and La proteins in green, and the cytoskeleton proteins in

Table I - ELISA values of Ro, La and HSP70 proteins.

	Control	*UV 1h*	*UV 2h*	*UV 3h*	*40°C*	*42°C*	*44°C*
Ro	134.4±0.2330	210.3±0.5925	290.3±0.4924	381.3±0.7647	381.3±0.7647	450.3±0.5115	471.4±0.5274
p <	vs	0.0052	0.0181	0.0008	0.0008	0.0141	0.0081
La	134.2± 0.1374	349.2± 3.048	392.1± 0.0690	410.8± 0.2948	389.9± 0.3167	430.7± 0.2656	471.7± 0.8337
p <	vs	0.0001	0.0262	0.0164	0.0103	0.0314	0.0001
HSP70	131.7± 0.4156	471.7± 0.8337	490.7± 0.2242	520.6± 0.1899	550.4± 0.1618	680.1± 0.1838	720.1± 0.1709
p value	vs	0.0250	0.0401	0.0144	0.0048	0.0117	0.0071

Significant p value <0.05

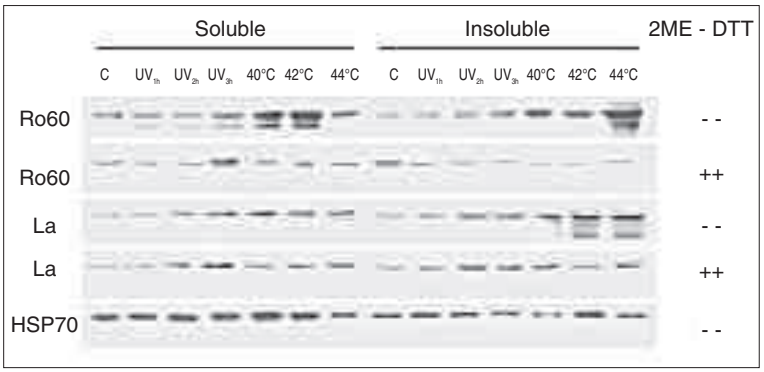

Figure 1 - Expression of Ro, La, HSP70 in control (c) and stressed HEp-2 cells studied by Western blot, that show aggregates of Ro soluble species and La insoluble (pellet) species, in reducing (2ME = bis-mercaptoethanol., DTT = Dithiothreitol) and non reducing conditions. HSP70 was included as housekeeping protein in the bottom.

red was done. The main result of this assay demonstrated that Ro and La ribonucleoproteins did not co-localize with the cytoskeleton proteins of control cells, however, under stress there was co-localization of Ro, La and HSP70 with tubulin and/or actin (Fig. 2).

The localization of Ro and La proteins in the cytoskeleton depends of HSP70

Taking in to account our previous findings, the interaction between the cytoskeleton and HSP70 and Ro/La ribonucleoproteins was further studied by crossed-immunoprecipitation. The results of this

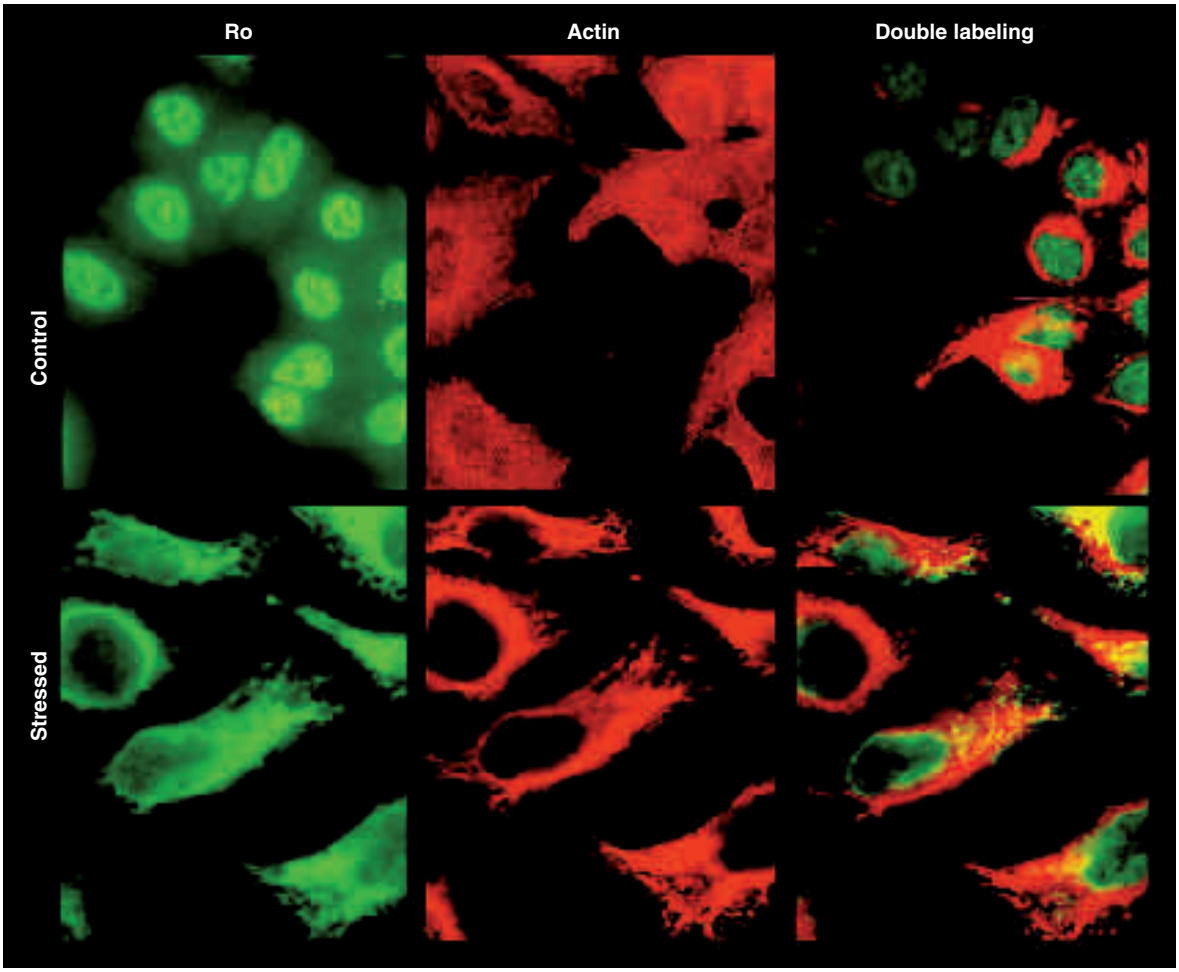

Figure 2 - Double fluorescence assay of Ro and La proteins double tagged in green, and the cytoskeleton proteins in red. A representative co-localization of Ro with actin in stressed HEp-2 cells.

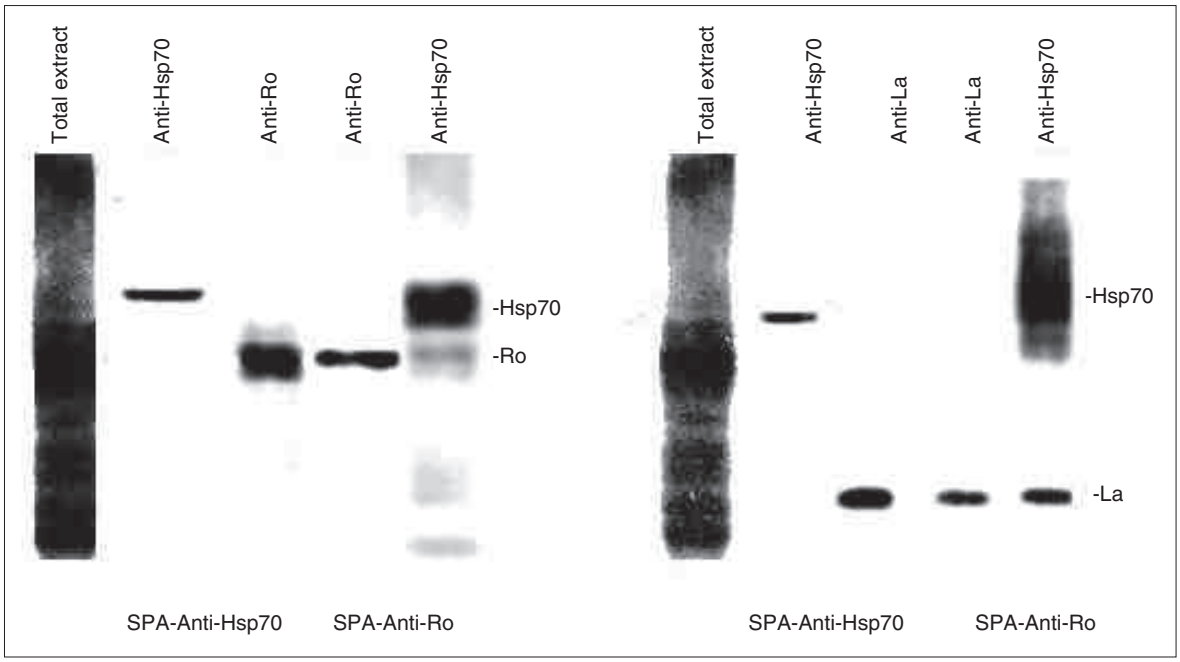

Figure 3 - Co-precipitation assays with anti-HSP70 linked to CNBr-4B Sepharose and co-precipitates were tagged with anti-Ro, anti-La or vice versa.

assay did not show precipitation of Ro and La by cytoskeleton proteins, however the HSP70 was co-precipitated by anti-actin and anti-tubulin antibodies, and in turn HSP70 precipitates simultaneously Ro/La and cytoskeleton proteins. This finding suggests that the association of Ro and La ribonucleoproteins with the cytoskeleton proteins are HSP70 dependent (Fig. 3).

DISCUSSION

Present studies address the molecular modifications of Ro and La ribonucleoproteins induced by stress. Main results of present studies demonstrated that the stress redistributes Ro and La ribonucleoproteins in cell compartments and induce conformational changes by self-aggregation.

Cellular stress is a physiological condition triggered by heat, glucose starvation, radiation and others. By stress the transcription of heat shock proteins (HSP) is activated; and HSP play a role in degradation, refurbishing and reactivation of damaged proteins (25). The stress induces changes of the cytoskeleton which is of particular importance because the actin-based cytoskeleton is a sensitive monitor of extra cellular stimuli (26, 27).

Is well established that stress increases the availability of intracellular antigens on cell surface, in consequence the autoantibodies trigger better antigens produced under cellular stress (28, 29), present results confirm this notion, and suggest that the Ro and La redistribution is associated to the cytoskeleton modification; another interesting observation suggest that Ro and La suffer conformational changes and aggregates form transitory complexes with the cytoskeleton proteins through HSP70. As support to our Ro ad La proteins tagged another experimental model have been demonstrated that under UV irradiation Ro and La are shuttled to cell membrane associated to calreticulin and HSP proteins, the transport is executed by the cytoskeleton (30-32).

Current work is of clinical importance because mimics stressful conditions like sunlight exposure that produce flare-ups in lupus, and increases the expression of Ro and La antigens on cell surface; this is followed by *in situ* formation of immune complexes of Ro/La and anti-Ro and/or anti-La antibodies that in turn trigger the skin lesions (29). For this reason it was important to define whether the UV and caloric stress induced conformational changes of Ro60 and La molecules that could affect its antigenicity. Present studies demonstrate that Ro60 and La self-aggregates under stress, this molecular modification was demonstrated previ-

ously *in situ* (1, 34, 35), Ro60 may form aggregates *in vitro*, and induce complexes with other proteins such as nucleolin, calreticulin, hnRNP K, and hnRNP the latter with chaperone activity (35, 36). Present investigation was carried out *in vivo*, and demonstrates that cell stress induce self-aggregation of Ro and La ribonucleoproteins, also shows association of these ribonucleoproteins with HSP70, this result suggest that HSP70 r may play a role as a carrier of Ro and La during their transit to cell membrane (37), another subrogate function of HSP70 may be in refurbishing the self-aggregates of Ro and La ribonucleoproteins. The importance of present studies is the experimental verification that stress produces self-aggregation Ro and La proteins and this conformational modification enhances their antigenicity. The antigenic expression in patients with autoimmune diseases, is important because favor the targeting of anti-Ro or anti La antibodies in tissues of Sjögren, SCLE and neonatal lupus (38). In conclusion, the cell stress redistributes Ro and La proteins whiting nucleo-cytoplasmic compartments. This redistribution is accompanied by self aggregation of Ro and La which interact with the cytoskeleton through HSP70. Finally the cell stress is an important factor for antigenic redistribution.

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SUMMARY

Ro and La antigens are of clinical interest in subacute cutaneous lupus erythematosus because skin lesions appear after UV irradiation, which induces the translocation of intracellular Ro and La ribonucleoproteins and trigger autoantibody production. Present studies address the question whether cellular stressors modify molecular characteristics and distribution of Ro60 and La proteins. To accomplish our goal HEp-2 cells were stressed by heat and UV irradiation and Ro and La expression was studied by indirect immunofluorescence and Western blot and crossed-immunoprecipitation using monoclonal anti-Ro/La or anti-HSP70 linked to CNBr-Sepharose 4B.

Results of present studies confirm that Ro60 and La were located in the nuclei of non stressed cells; however under stress, both ribonucleoproteins were redistributed within cytoplasm and nucleoplasm, interestingly the stress induces self aggregation of both ribonucleoproteins, as demonstrated the Western blot assays. Ro and La proteins interact with the cytoskeleton protein via HSP70.

In conclusion, the cell stress redistributes Ro and La proteins whiting nucleo-cytoplasmic compartments. This redistribution is accompanied by self aggregation of Ro and La which became associated with HSP70. Finally, the cell stress is an important factor for antigenic redistribution.

Parole chiave - Antigeni Ro-60, anticorpi anti-Ro, antigeni La, stress cellulare, ribonucleoproteine.
Key words *- Ro60 antigen, anti-Ro antibodies, la antigen, anti-La antibodies, cellular stress, ribonucleoproteins.*

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