Disparate Locations of the 52- and 60-kDa Ro/SS-A Antigens in Cultured Human Keratinocytes

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Anti–52- and anti–60-kDa Ro/SS-A (Ro) autoantibodies are produced by most patients with subacute cutaneous lupus erythematosus and neonatal lupus erythematosus and are thought to be pathogenic in these two disorders. To learn more about the epidermal antigens targeted by Ro autoantibodies, a panel of anti-52 and anti–60-kDa Ro antibodies was purified from human autoimmune sera and rabbit antisera and then used to: (i) determine the expression and location of the Ro antigens in human keratinocytes; (ii) clarify discrepancies in previous localization studies; and (iii) verify the existence of Ro autoantibodies that cross-react with the 52- and 60-kDa Ro antigens, as previously reported.

By immunoblot analysis these antibodies demonstrate that 52- and 60-kDa Ro proteins are expressed in normal human skin and cultured keratinocytes. By indirect immunofluorescence studies with cultured human cells, the anti–52-kDa Ro antibodies produce fine granular cytoplasmic fluorescence and less intense nuclear fluorescence, with apparent nucleolar sparing. The anti–60-kDa Ro autoantibodies produce weak cytoplasmic fluorescence and intense coarse granular nuclear fluorescence with apparent nucleolar sparing. We found distinct differences in the intracellular localization of the 52- and 60-kDa Ro autoantigens. This difference suggests that the 52- and 60-kDa Ro antigens may have independent cellular functions. Finding 60-kDa Ro antigen predominantly in the nucleus challenges the notion that the majority of the intracellular 60-kDa Ro antigen is complexed with the cytoplasmic hY RNA. Additionally, our failure to find a cross-reactive epitope on these two proteins indicates that the 52-kDa Ro antigen is probably a true immunogen and not merely a protein that cross-reacts with anti–60-kDa Ro autoantibodies, as others have suggested. ***Key words: lupus/skin/immunofluorescence. J Invest Dermatol 107:622–626, 1996***

Anti–52- and anti–60-kDa Ro/SS-A (Ro) autoantibodies are produced by the vast majority of patients with subacute cutaneous lupus erythematosus and neonatal lupus erythematosus (McCauliffe *et al*, 1994). Although there is substantial evidence that these autoantibodies are pathogenic in these two diseases (Lee *et al*, 1989; McCauliffe, 1995), the cellular function and location of their target antigens have remained controversial. Multiple studies have characterized the cellular location of Ro antigens by immunofluorescence techniques but with discrepant results. These discrepancies have arisen in part from differences in fixation techniques (Harmon *et al*, 1984; Peek *et al*, 1993), the use of non-human substrates (Harmon *et al*, 1984), and the utilization of human Ro autoimmune sera of varying specificities (Hendrick *et al*, 1981; Maddison *et al*, 1981; Harmon *et al*, 1984; Elenitsas *et al*, 1986; Lopez-Robles *et al*, 1986; Miyagawa *et al*, 1988). Recent studies, with standard fixation methods, human substrate, and more specific Ro antibodies, however, have also produced discrepant results (Bachmann *et al*, 1986; Ben-Chetrit *et al*, 1988; Slobbe *et al*, 1991; Peek *et al*, 1993; Casciola-Rosen *et al*, 1994; Kelekar *et al*, 1994; Peek *et al*, 1994). The interpretations of these data are even less certain because it has been reported that some Ro autoantibodies cross-react with the 52- and 60-kDa Ro proteins (Itoh *et al*, 1992).

Our aims in this work were to determine the expression and localization of 52- and 60-kDa Ro antigens in human keratinocytes. We also hoped to clarify the discrepancies reported in previous localization studies by using a panel of anti–52- and anti–60-kDa Ro-specific antibodies, and to determine whether previous inconsistencies may have resulted from cross-reacting antibodies.

MATERIALS AND METHODS

Isolation of 52- and 60-kDa Ro-Encoding Clones cDNA clones that encode the 52- and 60-kDa Ro antigens were isolated and used to make recombinant glutathione S-transferase Ro fusion proteins, as previously described (McCauliffe *et al*, 1994).

Rabbit Immunization New Zealand white rabbits were immunized with agarose-linked recombinant 52- and 60-kDa fusion proteins according to a well established protocol (Oettinger *et al*, 1992).

Antibody Purification

Human and Rabbit Anti-Recombinant 52- and 60-kDa Ro Antibodies Ro fusion proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred electrophoretically to polyvinylidine difluoride paper (Immobilon-P, Millipore Corp, Bedford, MA) with a semi-dry transfer unit (Semi-phor TE70, Hoefer Scientific Instruments, San

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Abbreviations: Ro autoantibody, anti-52- and anti-60-kDa Ro/SS-A autoantibody.

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Francisco, CA). The paper was then blocked with Blotto (Harlowe and Lane, 1988) for several hours, incubated with human Ro autoimmune sera or rabbit anti-sera overnight at 4°C, and then washed three times with a large volume of 1× phosphate-buffered saline (PBS) at room temperature. The bound antibodies were stripped from the polyvinylidine difluoride paper with 0.2 M glycine (pH 2.8) and immediately equilibrated to pH 8.0, with 1 M Tris, pH 9.5.

Human Anti-Native 60-kDa Ro Antibodies Twenty-four wells of a 96-well microtiter plate were coated with purified native bovine Ro (Immunovision, Springdale, AK) diluted to 8 U per ml in coating buffer (32 mM Na_2CO_3 and 68 mM $NaHCO_3$). These were incubated overnight at 4°C, blocked for 1 h (0.25% bovine serum albumin and 0.1% goat γ-globulin in 1× PBS), and incubated with a 1:5,000 dilution of high titer anti-native 60-kDa Ro containing autoimmune serum for 1 h. After two washes, the bound antibodies were stripped from each well with 0.2 M glycine, pH 2.8, for 5 min, and the pH was immediately equilibrated to pH 8.0, with 1 M Tris(hydroxymethyl)-aminomethane, pH 9.5. The antibodies from each well were pooled and concentrated (Spin-X UF concentrator, Costar, Cambridge, MA).

Enzyme-Linked Immunosorbent Assay (ELISA) Purified antibodies were tested against several recombinant proteins by ELISA, as previously described (McCauliffe *et al*, 1994).

Cell Extract Preparation A human skin extract was prepared from a freshly isolated plastic surgery specimen. The dermis was separated from the epidermis with 1 M NaCl, as previously described (Woodley *et al*, 1984). The epidermis was then snap-frozen in liquid nitrogen and pulverized. The pulverized epidermis was suspended in lysis buffer (50 mM NaCl, 0.3% Nonidet P-40, 10 mM dithiothreitol, 15 mM ethylenediamine tetraacetic acid) containing protease inhibitors [1.4 mM phenylmethanesulfonyl fluoride, pepstatin (2.5 μg/ml), leupeptin (0.5 μg/ml), and aprotinin (0.4 μg/ml)], and the supernatant fraction was collected by centrifugation. Wil-2 cell (ATCC CRL 8885) and cultured human keratinocyte extracts were similarly made by suspending $\times 10^{6-7}$ cells in 1 ml of lysis buffer followed by sonication with three 20-s bursts.

Immunoblot Analysis Immunoblot analysis was performed on the human skin, cultured keratinocytes, and Wil-2 cell extracts as previously described (McCauliffe *et al*, 1994). The Wil-2 human lymphoblastoid cell line served as a positive control as this cell line has previously been shown to express both the 52- and 60-kDa Ro mRNA (McCauliffe *et al*, 1994).

Keratinocyte Culture Neonatal foreskins were stored in Dulbecco's modified Eagle's medium-high glucose, with 1 mM sodium pyruvate, 2% fetal bovine serum, 5 μg ketoconazole per ml, 100 μg chloramphenicol per ml, 100 U penicillin per ml, 100 μg streptomycin per ml, 250 ng amphotericin B per ml, and 20 mM HEPES (pH 7.3), for less than 24 h. They were washed and trypsinized overnight at 4°C. The keratinocytes were resuspended in medium 154 with human keratinocyte growth supplement (Cascade Biologics, Inc, Portland, OR). Second passage keratinocytes were frozen in liquid nitrogen for later use. These keratinocytes were thawed and seeded onto eight-chamber Lab Tek slides (Nunc Inc., Naperville, IL). They were grown to 50% confluence.

Immunofluorescence The keratinocytes were fixed in 3% paraformaldehyde at 4°C for 15 min, followed by two brief washes with 1× PBS. They were permeabilized for 5 min with 0.1% Triton X in PBS, washed twice with 1× PBS, and then incubated for 1 h at 4°C with the purified Ro antibodies. Following another two 1× PBS washes, goat anti-human IgG conjugated to Texas Red and/or goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO) was added for 30 min at 4°C. The slides were washed twice, the chambers were removed, and the slides were coverslipped with polyvinyl alcohol mounting media. The slides were viewed with a conventional epifluorescence microscope, followed by confocal microscopy. Two confocal microscope systems were used. Images were collected with a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad Ltd, Hemel Hempstead, Herts, U.K.) mounted onto a Nikon Diaphot inverted microscope, with a 100× objective and a pinhole setting of 3 to maximize optical sectioning, at 0.8–0.9 μm. A double dichroic mirror directed a 488- and/or 568-nm argon–krypton laser to the sample. Excitation light was attenuated with a 1% neutral density filter to minimize photobleaching. The other confocal system used was a Zeiss invert LSM 410 with software version 3.50 (Gottingen, Germany) with a pinhole of 20 and a similar neutral density filter. The contrast and brightness adjustments were maintained at constant levels for all experiments and controls. Each digital image was obtained with a laser exposure time of 8 s, averaged over 4 s, to reduce noise. Confocal images were processed with Adobe Photoshop version 3.0 software and printed on an Epson Stylus color printer (Epson America, Inc., Torrance, CA).

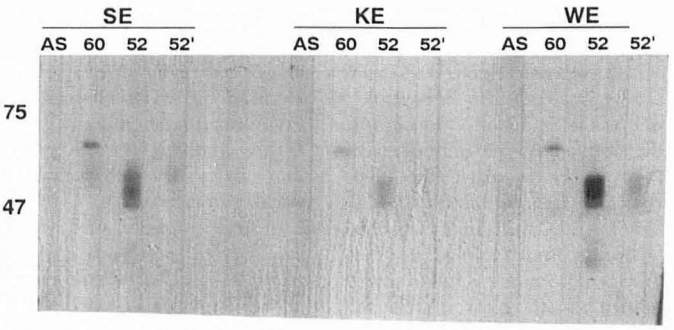

Figure 1. Extracts from human epidermis (SE), cultured keratinocytes (KE), and Wil-2 cells (WE) express 52- and 60-kDa Ro proteins as demonstrated by immunoblot analysis. Each extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidine difluoride paper, and then incubated with a 1:25 dilution of Ro-negative autoimmune control sera (AS), purified human anti-60-2 antibodies (60), and human anti-52-2 antibodies purified from two different autoimmune sera (52 and 52').

RESULTS

Multiple Types of Anti–52- and Anti–60-kDa Ro Antibodies Were Purified Rabbit antibodies were purified against full length recombinant 52- and 60-kDa Ro proteins. Human autoantibodies were affinity purified against two different recombinant 52-kDa Ro fragments [52-1b (amino acids 1–98) and 52-2 (amino acids 138–340)] and two different recombinant 60-kDa Ro fragments [60-2 (amino acids 139–326) and 60-4 (amino acids 410–538)]. Each of the four fragments had previously been shown to contain at least one major Ro epitope [52-2, 60-2, 60-4: (McCauliffe *et al*, 1994); 52-1b: personal unpublished observation). Human antibodies were also purified against the native 60-kDa Ro protein, which contains one or more epitopes not expressed by the recombinant Ro proteins (McCauliffe *et al*, 1994).

Cultured Keratinocytes and Human Epidermis Express the 52- and 60-kDa Ro Proteins The specificities of the purified antibodies were demonstrated by immunoblot **(Fig 1)** and ELISA **(Fig 2)**. These antibodies identified appropriately sized proteins in keratinocyte extracts prepared from surgical and cultured specimens, indicating that both Ro proteins are expressed in normal human skin and in cultured human keratinocytes, respectively. Multiple protein fragments were labeled with the anti–52-kDa Ro antibodies. Whether these fragments represent protein degradation, different forms of the 52-kDa Ro protein, or cross-reactive proteins is uncertain. The smear-like nature of the fragments, with a greater proportion of smaller fragments in the skin extract preparation, which takes longer to prepare than the cultured keratinocyte and Wil-2 cell extracts, would suggest that these fragments arise from protein degradation.

None of the Purified Ro Autoantibodies React with Both 52- and 60-kDa Ro Proteins There was no convincing evidence of cross-reactivity between the anti–52- and anti–60-kDa Ro antibodies by immunoblot. Furthermore, the ELISA results of our entire panel of purified Ro antibodies failed to validate the existence of the previously described cross-reactive epitope shared by the 52- and 60-kDa Ro proteins (Itoh *et al*, 1992).

Disparate Locations of 52- and 60-kDa Ro Proteins in Cultured Keratinocytes The cellular locations of the 52- and 60-kDa Ro proteins were determined by conventional epifluorescence and scanning confocal microscopy, including Z-series. The human anti–52-1b and 52-2 autoantibodies stained the cytoplasm in a fine granular pattern, with similar nuclear staining that was most

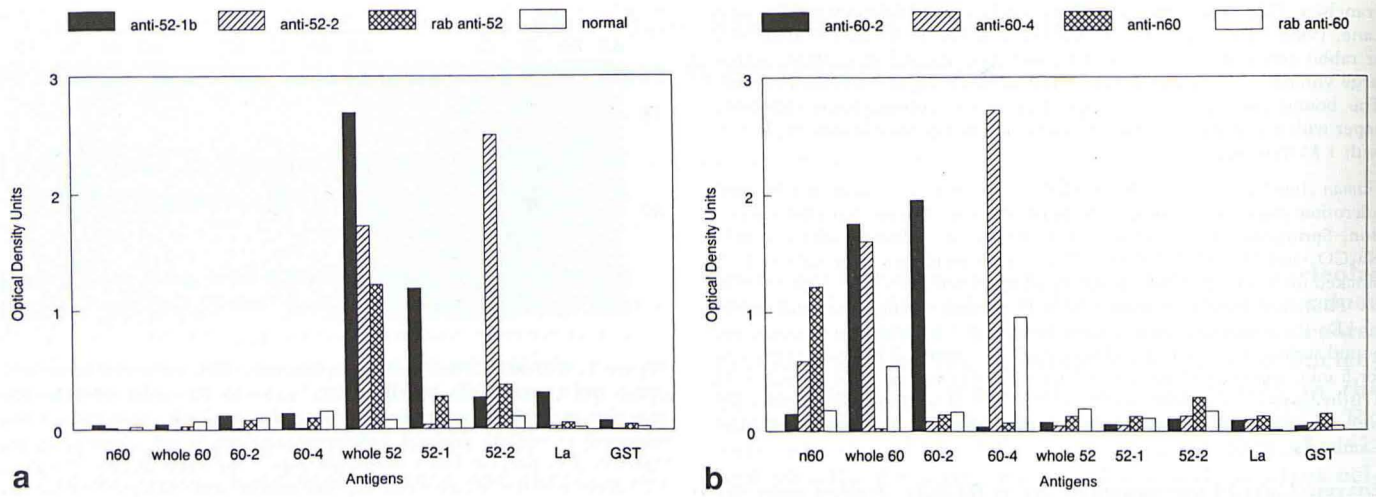

Figure 2. Purified Ro antibodies were specific for either the 52- or the 60-kDa Ro protein as demonstrated by ELISA. *(a)* Purified human anti–52-1b, anti–52-2, rabbit antisera raised against whole recombinant 52-kDa Ro (rab anti-52), and a 1:100 dilution of normal sera (normal) were reacted with a panel of antigens. *(b)* Purified human anti–60-2, anti–60-4, anti-native 60-kDa Ro (anti-n60), and rabbit antisera raised against the full-length recombinant 60-kDa Ro (rab anti-60), were similarly tested. Purified antigens: n60, native 60-kDa Ro; whole 60, full-length recombinant 60-kDa Ro; 60-2, recombinant 60-2 fragment (amino acids 139–326); 60-4, recombinant 60-4 fragment (amino acids 410–538); whole 52, full-length recombinant 52-kDa Ro; 52-1, recombinant 52-1 fragment (amino acids 1–178); 52-2, recombinant 52-2 fragment (amino acids 138–340); La, native La/SS-B autoantigen; and GST, recombinant glutathione S-transferase, which was a component of each recombinant fusion protein in this antigen panel.

apparent in cells that had not yet reached confluence. Cells that had reached confluence often demonstrated minimal nuclear staining **(Fig 3)**. Both the human anti–60-2 and 60-4 autoantibodies strongly stained the nucleus in a coarse granular pattern, with absent nucleolar and weak cytoplasmic staining **(Fig 4)**. The human anti-native 60-kDa Ro autoantibodies stained the cells similarly. Differences in 52- and 60-kDa Ro localization were confirmed by double immunofluorescence utilizing purified human and rabbit anti–52- and anti–60-kDa Ro antibodies **(Fig 5)**.

A similar analysis was performed on HEp-2 and HeLa cells, as several earlier studies did not detect differences in the locations of the 52- and 60-kDa Ro proteins in these cells (Ben-Chetrit *et al*, 1988; Slobbe *et al*, 1991; Peek *et al*, 1994). Cytoplasmic fluorescence was more pronounced with anti–52 than with anti–60-kDa Ro antibodies in these two epithelial cell lines. More nuclear fluorescence, however, was detected with anti–52-kDa Ro antibodies in HEp-2 and HeLa cells than in keratinocytes. This difference was most striking in cells that had reached confluence **(Figs 3, 6)**.

DISCUSSION

Localization Studies Reveal Disparate Locations of the 52- and 60-kDa Ro Antigens Ro cellular localization studies have produced inconsistent results for many years. Recent localization studies have found predominantly nuclear and minor cytoplasmic localization of both the 52- and 60-kDa Ro antigens in HEp-2 and HeLa cells (Ben-Chetrit *et al*, 1988; Slobbe *et al*, 1991; Casciola-Rosen *et al*, 1994; Peek *et al*, 1994). Another study, however, found predominantly cytoplasmic localization of 60-kDa Ro and mostly nuclear localization of 52-kDa Ro in HeLa and HEp-2 cells

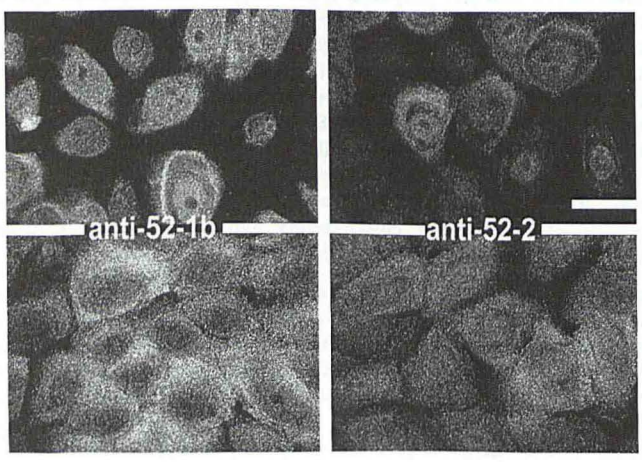

Figure 3. Confocal laser microphotographs reveal both nuclear and cytoplasmic localization of 52-kDa Ro. Immunofluorescence of cultured human keratinocytes with purified human anti–52-1b antibodies (*two panels on the left*) and anti–52-2 antibodies (*two panels on the right*). The *two uppermost panels* depict nonconfluent cells, and the *two lowermost panels* show cells that have reached confluence. *Scale bar*, 25 μm.

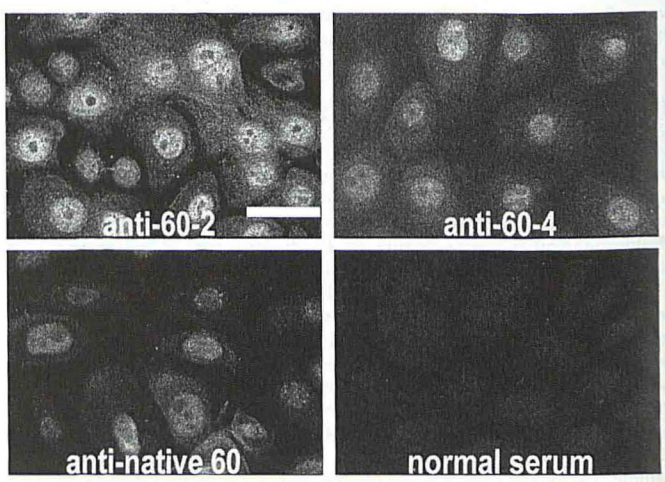

Figure 4. Confocal laser microphotographs reveal predominantly nuclear localization of 60-kDa Ro. Immunofluorescence of cultured human keratinocytes with purified human anti–60-2, anti–60-4, anti-native bovine 60-kDa Ro, and a 1:100 dilution of normal serum, as depicted. *Scale bar*, 25 μm.

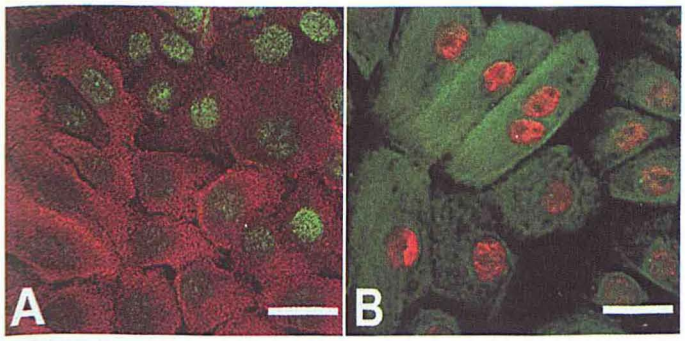

Figure 5. Confocal laser microphotographs of the double-immunofluorescent localizations of 52- and 60-kDa Ro confirm disparate locations of the 52- and 60-kDa Ro proteins in confluent keratinocytes. Double immunofluorescence of cultured human keratinocytes with *(A)* purified rabbit anti-60 kDa Ro antibodies (detected with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies) seen as *green* fluorescence, and human anti-52-2 antibodies (detected with Texas Red-conjugated goat anti-human antibodies) seen as *orange* fluorescence; and *(B)* purified rabbit anti–52-kDa Ro antibodies (detected with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies) seen as *green* fluorescence and human anti-60-2 antibodies (detected with Texas Red-conjugated goat anti-human antibodies) seen as *orange* fluorescence. *Scale bar*, 25 μm.

(Kelekar *et al*, 1994). Unfortunately most of these studies relied on a single monospecific autoimmune sera and/or antisera with anti–52 or anti–60-kDa Ro activity, and the specificity of these reagents was often not well defined. Monospecific anti–52-kDa Ro sera were defined by immunoblot analysis and may have contained anti–60-kDa Ro antibodies as this method does not detect all anti–60-kDa Ro antibodies (Boire *et al*, 1991).

Our immunofluorescence data are more convincing than any yet published because we used a panel of non–cross-reactive anti–52 and anti–60-kDa Ro- specific antibodies, all of which produced consistent yet disparate locations of the 52- and 60-kDa Ro antigens. Our predominantly nuclear localization of the 60-kDa Ro antigen is consistent with most of the earlier studies and a recent study that utilized monoclonal anti–60-kDa Ro antibodies (Veldhoven *et al*, 1995). We found, however, greater cytoplasmic localization of the 52-kDa Ro protein than did previous work. Most of the recent localization studies utilized "monospecific" 52-kDa Ro autoimmune sera (Ben-Chetrit *et al*, 1988; Slobbe *et al*, 1991; Casciola-Rosen *et al*, 1994) or partially purified rabbit antisera (Kelekar *et al*, 1994) that may have contained anti-native 60-kDa Ro or other autoantibodies that could produce nuclear fluorescence that overshadowed the cytoplasmic fluorescence generated by the anti–52-kDa Ro antibodies.

Overexpression of the 52-kDa Ro-encoding gene in HEp-2 and LTA-5 cells has demonstrated predominantly cytoplasmic localization of its protein product.[1] This same group had previously overexpressed the 60-kDa Ro protein and found predominantly nuclear localization (Keech *et al*, 1994).

We repeated our localization studies on HeLa and HEp-2 cells to make certain that our findings weren't unique to cultured keratinocytes. Our panel of anti–52- and anti–60-kDa Ro antibodies produced patterns of fluorescence in HeLa and HEp-2 cells similar to those in the cultured keratinocytes **(Fig 6)**, but diminished amounts of 52-kDa Ro in the nuclei of confluent HEp-2 and HeLa cells, as had been shown in confluent keratinocytes, were not demonstrated. This difference suggests that the 52-kDa Ro's cellular distribution may change as a result of keratinocyte differentiation induced by cell-cell contact, a change that may not occur in the immortalized HEp-2 and HeLa cells.

No Evidence That Ro Autoantibodies Cross-React with the 52- and 60-kDa Ro Molecules Itoh *et al* previously reported that a major denaturation-resistant epitope on the 52-kDa Ro protein is cross-reactive with Ro autoantibodies directed against an epitope expressed on the native 60-kDa Ro protein (Itoh *et al*, 1992). We have now shown that Ro autoantibodies purified against two different portions of the recombinant 52-kDa Ro (52-2 contains the previously described denaturation-resistant epitope), fail to react with the recombinant human 60-kDa Ro protein or the purified native bovine 60-kDa Ro protein **(Fig 2)**. Likewise, autoantibodies purified from the native 60-kDa Ro protein and two different fragments of the recombinant human 60-kDa Ro protein failed to react with the recombinant 52-kDa protein and protein fragments **(Fig 2)**. Whether the discrepancy between our data and that of Itoh *et al* resulted from differences in patient sera reactivities, source of Ro antigen, or methodology is uncertain. Other studies have also failed to reveal cross-reactivity of anti–52- and anti–60-kDa Ro autoantibodies (Ben-Chetrit *et al*, 1988; Peek *et al*, 1993; Keech *et al*, 1994; St.Clair *et al*, 1994).

52- and 60-kDa Ro Proteins Are Expressed in Cultured Human Keratinocytes and in Human Epidermis We have demonstrated that keratinocytes harvested directly from adult skin or from culture express both the 52- and 60-kDa Ro antigens. Our results differ from an earlier report that found no difference in the localization of 52- and 60-kDa Ro antigens in unprovoked cultured keratinocytes (Casciola-Rosen *et al*, 1994). This previous study used an anti–52-kDa Ro monospecific serum defined by immunoblot results, which may have contained anti–60-kDa Ro antibodies that masked the true localization of the 52-kDa Ro, as immunoblot analysis does not detect all anti-60 kDa Ro antibodies (Boire *et al*, 1991).

Previous studies have supported the hypothesis that ultraviolet B irradiation induces Ro antigens to translocate to the keratinocyte membrane where they might be bound by Ro autoantibodies and cause skin injury in neonatal lupus erythematosus and subacute cutaneous lupus erythematosus (LeFeber *et al*, 1984; Furukawa *et al*, 1990; Golan *et al*, 1992; Jones, 1992; Furukawa *et al*, 1994). Unfortunately, because these studies used poorly characterized monospecific Ro sera, it remains to be determined whether the 52-kDa Ro, 60-kDa Ro, or both proteins are so translocated. With our panel of Ro autoantibodies, we have found no evidence of 52- or 60-kDa Ro antigen expression on the surface of cultured keratinocytes before or 24 h after terrestrial-strength doses of

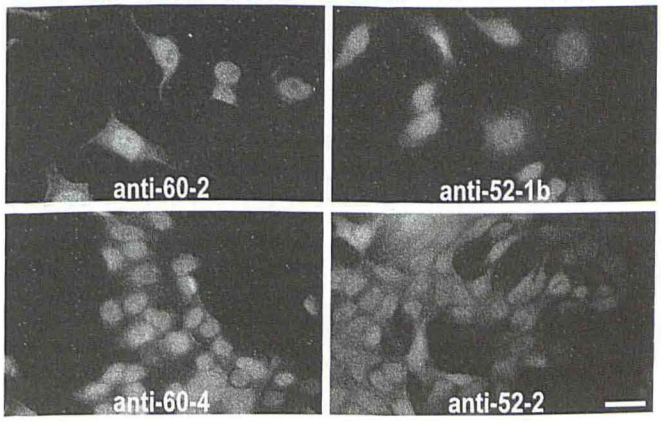

Figure 6. Epiluminescence microphotographs of the immunofluorescent localization of 52 and 60 kDa Ro antigens in HeLa *(two uppermost panels)* and HEp-2 cells *(two lowermost panels)* with anti–60- and anti–52-kDa Ro antibodies as indicated. *Scale bar*, 25 μm.

[1] Keech C, Gordon T, McCluskey, J: Cytoplasmic localization of the 52 kDa Ro/SS-A autoantigen in transfected mammalian cells and cloning of a mouse homolog. *Arthritis Rheum* 37(suppl):S172, 1994 (abstr.).

ultraviolet B irradiation (Yell, JA and McCauliffe, JP, unpublished observation).

We have demonstrated by immunoblot analysis that normal human epidermis expresses both the 52- and 60-kDa Ro antigens **(Fig 1)**. We have been unsuccessful, however, in localizing the 52- and 60-kDa Ro antigens in human skin biopsies by indirect immunofluorescence, despite having used different techniques on sections obtained from several donors (data not shown). We speculate that our inability to localize the antigens may be related to technical matters, but it is also possible that our skin biopsies came from individuals that produce low amounts of Ro antigen, e.g., Niimi *et al* (1995) have recently reported finding up to a 2,000-fold difference in 60-kDa Ro skin expression among individuals. We have not noted such a difference in the level of 52- and 60-kDa Ro antigen expression in cultured keratinocytes harvested from several donors.

It Is Likely That the Majority of Intracellular 60-kDa Ro Is Not Complexed with hY RNA The functions of the 52- and 60-kDa Ro proteins remain unknown. There are convincing data that the 60-kDa Ro protein associates with cellular hY RNA as a ribonucleoprotein complex. It has been argued that the 52-kDa Ro protein is also a component of this complex (Peek *et al*, 1993), but others dispute this claim (Slobbe *et al*, 1992; Kelekar *et al*, 1994). Our findings indicate that the majority of 52- and 60-kDa Ro proteins are not complexed together. We would, however, have to add that given some similarities in distribution, it is possible that some 52- and 60-kDa Ro molecules are components of the same ribonucleoprotein particles. Cell fractionation methods and hY RNA and Ro protein injection studies have demonstrated that the Ro ribonucleoprotein complex is located predominately in the cytoplasm (Peek *et al*, 1993, 1994). Our localization of the 60-kDa Ro protein predominantly to the nucleus would therefore indicate that only a minority of the total cellular 60-kDa Ro protein is complexed with cytoplasmic hY RNA. Peek *et al* (1993) have reported that the majority of 60-kDa Ro is in fact located in the cytoplasm, but autoantibodies cannot detect it because of epitope masking when the protein is part of the Ro ribonucleoprotein particles. It is possible that our immunofluorescence methods of fixation and/or permeabilization may have failed to preserve and/or unmask epitopes on the cytoplasmic form of 60-kDa Ro. This is less likely, however, as we have used antibodies reactive with at least three different 60-kDa Ro epitopes, and other investigators, using different immunofluorescence techniques, have also found a predominantly nuclear distribution of the 60-kDa Ro antigen (Lopez-Robles *et al*, 1986; Ben-Chetrit *et al*, 1988; Casciola-Rosen *et al*, 1994; Peek *et al*, 1994; Veldhoven *et al*, 1995).

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