Binding of Antibodies to the Extractable Nuclear Antigens SS-A/Ro and SS-B/La Is Induced on the Surface of Human Keratinocytes by Ultraviolet Light (UVL): Implications for the Pathogenesis of Photosensitive Cutaneous Lupus

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Autoantibodies to the non-histone nucleoprotein antigens SS-A/Ro, SS-B/La, and RNP are highly associated with photosensitive cutaneous lupus erythematosus (LE). In order to better understand the potential mechanisms of ultraviolet (UV) light on photosensitivity in patients with cutaneous LE, we designed immunopathologic in vitro and in vivo experiments to evaluate the effects of UV on the binding of such autoantibodies to the surface of human keratinocytes, one major target of immunologic damage in photosensitive LE. Short-term 2% paraformaldehyde fixation of suspensions of cultured human keratinocytes previously incubated with monoclonal antiserum probes enabled the detection of ENA expression on the cell surface by flow cytometry analysis. UVB light (280–320 nm) induced the binding of monoclonal antibody probes for SS-A/Ro and SS-B/La on keratinocytes in a dose-dependent pattern with maximal induction observed at the dose of 200 mJ/cm² UVB. Binding of SS-A/Ro, SS-B/La, and RNP antibody was augmented strongly, but binding of anti-Sm was very weak. In contrast, UVA (320–400 nm) light had no effect on the induction of binding of these antibody probes. Identical results were seen by standard immunofluorescence techniques. Hydroxyurea-treated keratinocytes showed similar induction of those antibodies by UVB irradiation, which suggested that ENA expression on cultured keratinocytes by UVB were cell-cycle independent. Tunicamycin, a inhibitor of glycosylation of proteins, reduced UVB light effect on the SS-A/Ro and SS-B/La antigen’s expression. These in vitro FACS analyses revealed that ENA augmentation on the keratinocyte cell surface was dose dependent, UV dependent, glycosylation dependent, and cell-cycle independent. In vivo ENA augmentation on the keratinocyte surface was examined in suction blister epidermal roofs. Specific antibody probes for SS-A/Ro, SS-B/La, RNP, and Sm bound to human keratinocytes in intact suction blister epidermis following UVB irradiation in vivo. Using three different protocols, we have demonstrated that antibodies to SS-A/Ro, SS-B/La, and U/RNP bind to UVB-irradiated human keratinocytes. We speculate that this antibody binding is an important inducer of antibody dependent keratinocyte damage in photosensitive cutaneous lupus. J Invest Dermatol 94:77–85, 1990

Photosensitivity is one of the major symptoms of systemic lupus erythematosus (SLE) [1]. Cutaneous lesions are frequently associated with ultraviolet light (UVL) exposure and are also aggravated by exposure to sunlight [2]. Some investigators have reported that the irradiation of previously uninvolved skin with multiples of a minimal erythema dose reproduces the morphologic and histologic changes of cutaneous LE [3–5]. The mechanisms involved in the reaction of the skin in LE following exposure to light are not well understood. Auto-antibodies may be related to the pathogenesis of cutaneous LE because immunoreactants deposit at the dermo-epidermal junction of the sun-exposed skin of LE patients [6]. These deposits can be induced experimentally by UVL [7], and UVL induces the release of nuclear antigens which bind with specific antibody in the skin [8].

Anti-SS-A/Ro and SS-B/La antibodies in sera are strongly associated with cutaneous LE, especially subacute cutaneous lupus erythematosus (SCLE) [9,10]. SCLE is a subset of cutaneous LE which is characterized by papulosquamous skin lesions, alopecia, and photosensitivity [9]. Recent studies on the pathogenesis of photosensitive cutaneous LE have focused on the close association of antibodies to the antigen SS-A/Ro with development of clinical disease.
The epidermal keratinocyte, especially the basal cell, is the major target of immunologic damage in photosensitive lupus. Recently we reported that UVL-irradiated, cultured human keratinocytes bind IgG antibodies from the sera of LE patients with either monospecific anti-SS-A/Ro, anti-RNP, or anti-Sm activities, which implies that these antigens have been made accessible on the cell surface by UVL irradiation [12]. The experiments reported in this paper verify the prior observations by three different protocols for detection of anti-SS-A, SS-B, and RNP binding to keratinocytes irradiated in vitro and in vivo, and determine that this is a UVV-dependent process. The effect of UVL on induction of binding of anti-Sm was significantly less. The modulation of this antigen on the cell membrane of keratinocytes in vitro by UVL has led to the hypothesis that binding of anti-SS-A/Ro antibodies to keratinocytes in the skin may be the immunologic trigger of the photosensitive skin disease seen in SCLE. The possible association of SS-A/Ro and SS-B/La antigens in an intracellular molecular complex is inferred from studies in cell culture [13,14] and the strong co-association of antibodies to both antigens in patients with photosensitive LE [10,15] has increased interest in the possible cooperative role of anti-SS-A/Ro and anti-SS-B/La antibodies in photosensitive LE.

Based on the postulated role of these antibodies in keratinocyte damage in photosensitive cutaneous LE, we designed the following immunopathologic experiments to evaluate the in vitro and in vivo effects of UVL on ENA expression on keratinocytes by indirect immunofluorescence (IF) and flow-cytometric analysis.

MATERIALS AND METHODS

Culture Medium The basic medium for human keratinocyte culture used in the present study consists of MCDB153 (Clonetics, San Diego, CA) supplemented with 10 ng/ml epidermal growth factor (Collaborative Research, Boston, MA), 5 μg/ml insulin (Sigma, St. Louis, MO), 1.4 μM hydrocortisone (Sigma), 0.1 mM ethanolamine (Sigma), and 0.1 mM phosphoethanolamine (Sigma).

Cell Culture Culture of human keratinocytes was performed according to our previous report [12]. Neonatal human keratinocytes from neonatal foreskins obtained from circumcisions were grown in culture by the method of Boyce and Ham [16]. Keratinocytes were plated into 25-cm² plastic flasks (Corning Glass Works, Corning, NY) in low-calcium (0.1 mM) serum-free medium. Primary cultures were expanded in the first passage and frozen in aliquots. In the present studies, second-passage cells were used.

The keratinocytes from neonatal foreskins formed discrete, uniform colonies of undifferentiated, noncornified, cuboidal cells resembling epidermal basal cells. Staining with a monoclonal antibody specific for epidermal basal cells was positive in the vast majority of these cells from neonates [17]. Immunofluorescent staining with antibodies to keratin, to Langerhans cells from neonatal foreskins obtained from circumcisions were ethanolamine and anti-Sm antiserum bound to 43-Kd or 60-Kd proteins. Taken together with passive hemagglutination, ID and CIE, these results confirmed the antibody specificity and indicated that anti-SS-A/Ro and anti-SS-B/La activity were the only detectable antibodies and that these sera were appropriate monospecific probes, considering the sensitivity of the IF methods used in this investigation. In experiments where anti-SS-A/Ro and anti-SS-B/La activity were simultaneously examined, these probes were combined in equal amounts. Monospecific anti-RNP antiserum and anti-Sm antiserum were kindly provided by the Department of Rheumatology, University of Colorado School of Medicine. Monoclonal anti-single-stranded (ss) DNA antibody and monoclonal anti-ss/double-stranded DNA antibody were obtained from autoimmune MRL/Mp-1pr/1pr and BXS3 mice by the hybridoma technique [20].

UV Light Irradiation Before irradiation, the medium in Lab-Tek chamber slides (Miles, Naperville, IL) or 60-mm plastic dishes was replaced with phosphate-buffered saline (PBS) free of any photoactive compounds. At least one dish was not exposed to UV light (but was handled in the same fashion as the irradiated cells), whereas the remainder were exposed separately to varying doses of UVA or UVB produced by several different light sources. As a UVA light source, a Hoya F24.12BL lamp (mainly UVA light peaking at 365 nm) was used. The irradiance of UVA (320 to 400 nm) measured by an International Light (Newburyport, MA) IL1350 radiometer was 3.13 mW/cm² at a distance of 6.5 cm, and the UVB irradiance was 0.01 mW/cm² as measured through both plastic dish cover and 2-mm-thick slide glass.

As our major UVB light source we used a National Sun Lamp FS20 (mainly UVB light peaking at 305 nm) containing two bulbs.

[10,11].
The UVB irradiance (280–320 nm) measured by IL1350 radiometer was 0.41 mW/cm² at a distance of 6.5 cm. Unfiltered FS 20 lamps were used in the present study and, in some cases, glass filters were employed to block UVB and UVC. In addition, in some experiments we used a SOL 3 solar simulator (Dermalight 2001 with h 2 filter (295 - 400 nm), Munich, Germany), and added clear glass to block UVB. This source contains a mixture of UVB and UVA without UVC, and additional filtering removes the UVB component of the emission.

Indirect IF methods Previous techniques developed in this laboratory have facilitated the separation of cell-surface, cytoplasmic, and nuclear-antigen staining patterns by different protocols of antibody incubation and fixation [12]. In these studies, cell-surface antibody binding was measured by incubating viable cells with antibody at 4°C for 1 h, followed by rapid fixation. Exclusion of antibody from cytoplasm and nucleus in cells with granular cell-surface staining was verified visually. Alternatively, if the cells were fixed (permeabilized) before adding antibody to ENA, cytoplasmic and nuclear staining patterns were seen. Fixation before addition of antibodies to DNA produced nuclear staining patterns. Granular membrane staining was seen in viable cells.

A second-passage keratinocyte suspension, 1.5 ml (625 X 10⁴ cells) was plated in each well of Lab-Tek chamber slides and cultured for 4 to 5 d in supplemented MCDB153 medium. In most experiments, 24 h after UV-light irradiation, each specimen on a glass slide was washed with PBS and incubated with diluted (1:100) antisera or diluted normal human serum (NHS) at 4°C for 1 h. Subsequent fixation and staining with fluorescence-conjugated second antibody were performed as previously described [21]. At least 500 cells were counted and the % positivity was determined by subtracting values from unirradiated cells from the values for radiated cells. Each test was run in duplicate.

Flow-Cytometry Analysis A FACs technique was developed which allowed identification of cell-surface antibody binding but limited subsequent internalization. Second passage cells were cultured in supplemented MCDB153 medium on 60-mm plastic dishes for 4 to 5 d. When the cells grew to 60% to 70% confluency, the cells were irradiated with UV light. Twenty-four hours after irradiation, the cells were harvested and stained for FACS analysis as previously described [22]. In each experiment, negative controls included cells not irradiated but stained with the same antiserum plus second antibody and also irradiated cells stained with NHS and second antibody.

Cell Viability Cell viability was tested by trypan blue dye exclusion test and by the acridine orange/ethidium bromide (AO/EB) test. Determination of cell viability with AO/EB staining was carried out according to the method of Parks et al [23] on second passage cells grown on Lab-Tek chamber slides.

Chemical Agents Hydroxyurea (1, 2, and 5 mM, Sigma) was used for detection of cell-cycle dependency. Hydroxyurea synchronizes cells at the G1/S boundary [24]. Cell cultures were preincubated with this agent for 4 or 24 h prior to irradiation, were maintained at the same concentrations of hydroxyurea for an additional 24 h, and then were prepared for flow-cytometry analysis.

Tunicamycin (2 μg/ml, Sigma) was added to the cultures for 0, 4, or 24 h prior to irradiation, and maintained in culture for 24 h after irradiation for determination of the role of glycosylation in antigen expression [25]. Tunicamycin-treated cells were also prepared for flow-cytometry analysis. 2-deoxy-D-glucose (1 mM, Sigma) was added in a similar way as a glycolysis inhibitor [26], and cytochalsin B (18 μM, Sigma) was added as a microfilament inhibitor [27].

In Vivo UVB Light Irradiation The surfaces of the forearms of adult male volunteers were exposed to 100 or 200 mJ/cm² UVB light, and 24 h later suction-blisters epidermal roofs were obtained from the UVB-irradiated sites and non-irradiated sites. Details of suction-blisters formation were described recently [19]. Intact epidermal roofs were washed with PBS and incubated with monospecific antisera at 4°C overnight. Then, specimens were washed with PBS and embedded in OCT compound (Miles). Four-μm cryostat sections were incubated with FITC-labeled rabbit F(ab')₂ anti-human IgG (DAKO), diluted 1:100 in PBS, at RT for 30 min. After washing and drying, one drop of PPD mounting medium [21] was placed over the stained specimens. Whole epidermal sheets stained for IF examination were mounted with PPD medium on glass slides. Stained specimens were observed by epifluorescence microscopy. In this protocol, skin is irradiated in vivo, whole intact epidermal roofs are incubated with antibody in vitro, and then the presence of cell surface or nuclear staining is determined after sectioning and staining of epidermis.

Statistical Analysis Student t test and the t test for paired samples were used and probability values less than 0.05 were considered significant.

RESULTS
First, to confirm our previous report [12], we examined the binding of antibody probes to the surface of keratinocytes cultured on Lab-Tek chamber slides as an indication of cell-surface antigen expression. As shown in Fig 2A, expression of certain nuclear antigens was observed on the cell surface of cultured keratinocytes irradiated with UVB at the dose of 200 mJ/cm². Anti-SS-A/Ro, anti-SS-B/La, and anti-RNP antisera showed strong granular positive staining on cell surfaces. These antisera showed granular cell surface staining in 23% to 29% of cells counted (Table I). In contrast, the fluorescence intensity and the percent of positive staining cells produced by the anti-Sm antiserum were relatively weaker than that of antisera to the other extractable nuclear antigens. Monoclonal anti-DNA antibodies showed no specific staining of cultured keratinocytes irradiated with 200 mJ/cm² of UVB. UVA light induced no augmentation of SS-A/Ro or SS-B/La expression at doses of 100 mJ/cm² to 5 J/cm².

The more quantitative method of flow-cytometry analysis was designed to better characterize UVB-induced nuclear antigen expression on the surface of cultured keratinocytes. Acetone or formaldehyde fixation of keratinocytes produced poor results because of high background staining. However, fixation of keratinocytes by short-term 2% paraformaldehyde solution after incubation with primary antibody facilitated the detection of SS-A/Ro and SS-B/La expression on the surface of cultured keratinocytes irradiated with UVB light. In initial experiments, a combination of anti-SS-A/Ro and anti-SS-B/La probes were used simultaneously. Fine granular staining was seen after short-term incubation with anti-Ro/La or anti-RNP on the surface of keratinocytes irradiated with UVB. Viability of keratinocytes irradiated with 100 mJ/cm² and 200 mJ/cm² UVB was 94% - 96% after staining. However, 500 mJ/cm² UVB produced 15% - 20% dead cells. These cells appear as a broad band of low frequency but high intensity to the right of the positive-staining peak. Binding of the anti-Ro/La antibody probe was augmented in a dose-dependent pattern (increasing percent positive cells at increasing UVL doses) and IF intensity was 92.3, 92.8, and 126.4 as mean channel number at the doses of 50, 200, and 500 mJ/cm² (Fig 3). Table II shows the induction of IgG binding to the keratinocytes using probes with SS-A/Ro and SS-B/La activity. With 20 and 50 mJ/cm² of UVB, 9.3% and 9.2% of keratinocytes bound IgG from the anti-SS-A/Ro plus SS-B/La positive probe. At the dose of 200 mJ/cm² of UVB, the highest percentage positivity was seen (24.6%). Similar results were obtained in the cells stained with the monospecific anti-SS-B/La antiserum and with the monospecific anti-SS-A/Ro antiserum used separately. Since the degree of UVL-augmented antibody binding varied with different cell sources, the cell source was carefully selected and cells from the same source were used in each experiment. Based on these results, 200 mJ/cm² of UVB was used as the optimal irradiation dose for the following experiments with flow cytometry analysis.

Table III shows the percentage of positive keratinocytes obtained with anti-SS-A/Ro after irradiation with three different light sources, with and without filtration through window glass. Filtra-
Figure 2. Staining with monospecific antisera of irradiated keratinocytes at 24 h post irradiation. (A) anti-SS-A/Ro antiserum; (B) anti-SS-B/La antiserum; (C) anti-RNP antiserum; (D) normal human serum. Cells were cultured on Lab-Tek chamber slides for 4 to 5 d and irradiated with 200 mJ/cm² UVB. Magnification ×400. Nuclei are counter-stained with propidium iodide (PI). In panels A to C, cell surface and cytoplasmic granular staining is shown by the white arrow and nuclear diffuse or granular staining is shown by white arrowheads. In panel C, indicating anti-RNP antiserum staining of cells, much more nuclear staining is seen.

Table 1. UVB Light-Induced Augmentation of Nuclear Antigens on the Surface of Cultured Keratinocytes

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Expression</th>
<th>Qualitative</th>
<th>Quantitative (%)</th>
<th>p Value (versus Sm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-A/Ro</td>
<td>+</td>
<td>+</td>
<td>25.4 ± 2.3</td>
<td>0.001 &lt; p &lt; 0.01</td>
</tr>
<tr>
<td>SS-B/La</td>
<td>++</td>
<td></td>
<td>23.6 ± 2.8</td>
<td>0.002 &lt; p &lt; 0.05</td>
</tr>
<tr>
<td>SS-A/Ro and SS-B/La</td>
<td>++</td>
<td></td>
<td>25.3 ± 1.8</td>
<td>0.001 &lt; p &lt; 0.01</td>
</tr>
<tr>
<td>RNP</td>
<td>++</td>
<td></td>
<td>28.2 ± 3.4</td>
<td>0.001 &lt; p &lt; 0.01</td>
</tr>
<tr>
<td>Sm</td>
<td>+</td>
<td></td>
<td>13.5 ± 1.7</td>
<td>0.001 &lt; p &lt; 0.01</td>
</tr>
<tr>
<td>dsDNA/ssmDNA</td>
<td>–</td>
<td></td>
<td>up to 1.0</td>
<td></td>
</tr>
<tr>
<td>ssDNA</td>
<td>–</td>
<td></td>
<td>up to 1.0</td>
<td></td>
</tr>
<tr>
<td>NHS</td>
<td>–</td>
<td></td>
<td>up to 1.0</td>
<td></td>
</tr>
</tbody>
</table>

* Grading reflects fluorescent intensity on a scale of 0 to 3 plusses according to our previous report [12]. The results represent the mean percentage (± one SEM) of positive cells from four experiments. Binding of each antibody to ENA was significantly higher than binding of NHS. Binding of SS-A/Ro, SS-B/La, and RNP were each significantly higher than that of Sm.
tion through window glass removes more than 95% of the UVB radiation. Because UVC is a contaminant (0.4% of total UVB dose) of the output of FS bulbs, the solar simulator was used to produce irradiation free of UVC, containing only long-wavelength UVA and UVB. FS20 and solar simulator radiation containing 200 mJ/cm² of UVB produced maximal SS-A/Ro induction (experimental conditions I and III, respectively), compared to control (experimental conditions VII). Filtration of FS20 radiation through window glass (experimental conditions II) reduced UVB dose by 97% and reduced the positive staining (experimental conditions III) to baseline levels (experimental conditions VII). High levels of UVA had no effect (experimental conditions V and VI). Even high levels of UVA added to intermediate (experimental conditions IV) or high (experimental conditions III) doses of UVB did not produce any augmentation of the UVB effect. No statistically significant synergy of UVA and UVB was seen with variable levels of UVA and high UVB (experimental conditions I and III). UVC was not crucial to induction of positive staining, as is seen by comparing experimental conditions I and III. The induction of SS-A/Ro positivity was clearly UVB dependent, and high levels of UVB alone were not effective. Combinations of UBV plus UVA did not significantly augment the effect of UVB alone.

Table IV shows the percentage of positive keratinocytes produced by the various antibody probes to nuclear antigens. UVB-induced apparent expression of SS-A/Ro and SS-B/La antigens on the surface of over 20% of cultured cells. In contrast, RNP antigen expression was positive but in a lower percentage of cells than SS-A/Ro and SS-B/La expression. Sm antigen expression was positive in a low percentage of cells, almost the same as the baseline condition.

As a next step, cell-cycle dependency was examined (Table V). Hydroxyurea at the 5-mM and 20-mM doses did not inhibit SS-A/Ro and SS-B/La antigen expression. When cells were pre-incubated with hydroxyurea (20 mM) for 4 or 24 h prior to irradiation, followed by hydroxyurea for 24 h after irradiation, similar results were obtained. Thus UVL induction of cell-surface binding of antibody probes did not require DNA synthesis or mitosis. Since a previous study showed that binding of SS-A/Ro probes following UVL was protein synthesis dependent [12], we felt further analysis of the effects of other metabolic inhibitors was necessary.

The effect of tunicamycin on SS-A/Ro and SS-B/La antigen expression was determined for evaluation of the role of glycosylation in UVB-induced antigen translocation to the surface of cultured keratinocytes. Figure 4 shows the flow-cytometry analysis of UVB-irradiated keratinocytes incubated with tunicamycin. UVB-irradiated keratinocytes incubated with tunicamycin for 24 h after UVB irradiation (Fig 4B) showed similar binding of the antibody containing both SS-A/Ro and SS-B/La probes, as did irradiated cells without tunicamycin treatment (Fig 4A). However, tunicamycin treatment for 4 h prior to UVB irradiation and for 24 h after the irradiation reduced the augmentation in half (Fig 4C). As shown in Fig 4D, keratinocytes pretreated with tunicamycin for 24 h prior to UVB irradiation showed little augmentation of SS-A/Ro and SS-B/La antigen expression. These results demonstrate that inhibitors of glycosylation, if applied early enough prior to irradiation, block the subsequent binding of specific antibody probes for SS-A/Ro and/or SS-B/La on irradiated keratinocytes.

Table VI shows the effect of metabolic inhibitors on anti-SS-A/Ro and anti-SS-B/La binding to the cultured keratinocytes irradiated with UVL. Inhibition of glycolysis (deoxyglucose) and glycosylation (tunicamycin) produced significant inhibition of binding of anti-SS-A/Ro and SS-B/La following UVL irradiation. Microfilament inhibition (cytochalasin) also inhibited this response.

The results of in vivo irradiation of intact human skin on binding

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### Table II. Flow-Cytometric Analysis: UVB Light Dose-Dependent Induction of Binding of SS-A/Ro and SS-B/La to the Surface of Cultured Keratinocytes Following UVB

<table>
<thead>
<tr>
<th>Dose of UVB light (mJ/cm²)</th>
<th>% positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (n = 5)</td>
<td>2.5 ± 1.9*</td>
</tr>
<tr>
<td>20 (n = 2)</td>
<td>9.3 ± 1.9</td>
</tr>
<tr>
<td>50 (n = 3)</td>
<td>9.2 ± 1.8</td>
</tr>
<tr>
<td>100 (n = 4)</td>
<td>13.6 ± 5.3</td>
</tr>
<tr>
<td>200 (n = 6)</td>
<td>24.6 ± 2.4</td>
</tr>
<tr>
<td>500 (n = 2)</td>
<td>23.5 ± 4.4</td>
</tr>
</tbody>
</table>

* The results represent the mean percentage ± 1 SEM.
The binding of antibody
This report verifies the original observation of LeFeber et al [12] some cells with tmd cell surface at the interface between adjacent cells cytoplasmic staining and areas of linear staining which appear at the atinocyte damage in photosensitive cutaneous lupus. The implica­ tion of this cell-surface binding of antibodies specific for ENA (but by UVL
serum probe containing
irradiated skin incubated with anti-SS-A/Ro and
many cells with bright nuclear staining permeabilization during blister formation. Nuclear counterstaining with PPD appears as dull staining. Only a few epidermal cells show positive bright fluorescence. On the other hand, panel B from UVL-irradiated skin incubated with anti-SS-A/Ro and SS-B/La shows many cells with bright nuclear staining (white arrowhead), many cells with diffuse granular cytoplasmic staining (white arrows), and some cells with faint linear or granular cell surface staining (black arrow). Since the antibodies were added to intact viable epidermis in organ culture, nuclear and cytoplasmic staining are believed to result from active internalization. Panels C and D show extensive cytoplasmic staining and areas of linear staining which appear at the cell surface at the interface between adjacent cells (black arrow, C and D). If normal human serum or anti-DNA antibodies were used in place of the antibody probes for SS-A/Ro and SS-B/La, no cyto­ plasmic, nuclear, or cell-surface staining was seen. An occasional diffuse nuclear staining of basal cells, which was interpreted as damage due to suction blister formation, was observed.

DISCUSSION
This report verifies the original observation of LeFeber et al [12] that antibodies specific for SS-A/Ro antigen were induced to bind to the cell surface of cultured human keratinocytes by UVL, a result of great potential importance to the induction or modulation of keratinocyte damage in photosensitive cutaneous lupus. The implication of this cell-surface binding of antibodies specific for ENA (but not for anti-DNA antibodies) is that these antigens are translocated by UVL to the cell membrane, where they bind IgG autoantibodies. The binding of antibody to keratinocyte cell membranes may induce cell damage, thereby producing the keratinocyte lysis character­ istic of photosensitive lupus.

The present study presents several important improvements to the original report [12], which greatly improve our confidence in the results and its implications. Three different test systems were used to show that antibody probes bind to keratinocytes following UVL: immunofluorescence of cultured human keratinocytes, FACS analysis of cultured keratinocytes trypsinized into suspension, and immunofluorescence of intact epidermis irradiated in vivo and then incubated with antibody probes in vitro. Analysis with a panel of monospecific probes for ENA (Ro, La, RNP, Sm) was used, and the specificity of the SS-A/Ro and SS-B/La antibody probes was verified by immunoblotting. Combination of visual IF and FACS facilitated analysis of the metabolic modulation of this apparent antigen translocation and subsequent antibody binding. This is an active event, dependent on protein synthesis, glycosylation, and/ or glycolysis, and microfilament function; cell-surface antibody binding can be seen in cells in all phases of the cell cycle, but can be blocked by protein-synthesis inhibitors.

In irradiated cell cultures or in irradiated intact epidermis incubated with antibody in vitro, antibody probes bound to the surface of keratinocytes, but were also seen to bind to cytoplasm and nucleus. A similar pattern of antibody localization was seen in our previous experiments in human skin grafted onto a nude mouse which was injected with anti-SS-A/Ro, and also in skin biopsies from SLE patients [28,29]. We interpret these results to indicate that internalization of antibody bound to cell surface in vitro and in vivo sometimes leads to nuclear internalization [29]. Additional experiments to characterize this antibody internalization are underway.

The significance of antibodies specific for the SS-A/Ro and SS-B/La antigens in the pathogenesis of cutaneous LE is hotly de-

Table III. Flow-Cytometric Analysis of Cultured Keratinocytes Irradiated with UVL*

<table>
<thead>
<tr>
<th>Experimental combination</th>
<th>UV</th>
<th>Light source</th>
<th>Dose (mJ/cm²)</th>
<th>Filter</th>
<th>% positive cells (stained with anti-SSA/Ro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A+B</td>
<td>FS 20</td>
<td>158 (A)</td>
<td>None</td>
<td>24.5 ± 3.7</td>
</tr>
<tr>
<td>II</td>
<td>A+B</td>
<td>FS 20</td>
<td>200 (B)</td>
<td></td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>III</td>
<td>A+B</td>
<td>Solar simulator</td>
<td>158 (A)</td>
<td>window glass</td>
<td>26.5 ± 4.8</td>
</tr>
<tr>
<td>IV</td>
<td>A+B</td>
<td>Solar simulator</td>
<td>10 (B)</td>
<td>UVC filter</td>
<td>12.5 ± 2.5</td>
</tr>
<tr>
<td>V</td>
<td>A</td>
<td>F24.12 BL</td>
<td>5000 (A)</td>
<td>UVC filter</td>
<td>4.5 ± 1.9</td>
</tr>
<tr>
<td>VI</td>
<td>A</td>
<td>F24.12 BL</td>
<td>1000 (A)</td>
<td>none</td>
<td>4.2 ± 1.7</td>
</tr>
<tr>
<td>VII</td>
<td>non</td>
<td>Control</td>
<td>none</td>
<td>non</td>
<td>3.5 ± 2.0</td>
</tr>
</tbody>
</table>

* Results represent the mean ± 1 SEM of at least three experiments.

† Using FS 20, UVC is a contaminant representing 0.4 % of UVB dose.

‡ 0.001 < p < 0.01.

§ 0.01 < p < 0.02.

\( 0.02 < p < 0.05. \)

Table IV. Binding of Antibody Probes on the Surface of Cultured Keratinocytes by Flow-Cytometry Analysis*

<table>
<thead>
<tr>
<th>Antibody Specificities</th>
<th>% Positive Cells</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versus RNP</td>
<td>Versus Sm</td>
<td></td>
</tr>
<tr>
<td>SS-A/Ro and</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS-B/La</td>
<td>24.5 ± 3.7</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>SS-A/Ro</td>
<td>21.8 ± 2.9</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>SS-B/La</td>
<td>20.4 ± 3.6</td>
<td>n.s</td>
</tr>
<tr>
<td>R N P</td>
<td>11.2 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Sm</td>
<td>1.5 ± 2.6</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Negative Control*</td>
<td>up to 1%</td>
<td></td>
</tr>
</tbody>
</table>

* Results represent the mean ± 1 SEM of at least three experiments.

† Normal human serum.
The UV effects on cell-surface antigen translocation were not dependent on cell cycle, DNA synthesis, or mitosis. Interestingly, tunicamycin pretreatment markedly reduced the expression of SS-A/Ro and SS-B/La antigens on UV-irradiated keratinocytes, whereas tunicamycin treatment after UV irradiation had no effects on antigen translocation. Tunicamycin inhibits dolichol pyrophosphate mediated glycosylation, affecting the glycosylation of asparaginyl residues specifically [39,40]. Although tunicamycin is reported to inhibit protein synthesis of fibroblasts, depending on its concentration, tunicamycin at the concentration utilized in this study, inhibited protein synthesis of human keratinocytes by less than 10% [41]. From these results, we suggest that the glycosylation process before UVB irradiation is essential for SS-A/Ro and SS-B/La antigen expression on cultured keratinocytes. Similar results were seen with deoxyglucose and cytochalasin. The binding of ENA-specific antibody probes to keratinocytes following UVL is apparently an active, complex process, requiring protein synthesis, glycosylation, and microfilament function. We believe that this is most consistent with active energy-dependent translocation of antigen to the cell membrane. Of course, an alternative hypothesis is that glycosylation and microfilament activity are necessary to translocate another protein, which facilitates ENA translocation.

We recently reported an investigation of binding of anti-SS-A/Ro and anti-SS-B/La to keratinocytes following incubation with estradiol-beta (another trigger factor in lupus) [22]. It is interesting that intracellular translocation of nucleoproteins by estradiol has been reported in another test system [22].

In vivo induction of binding of antibodies to ENA on keratinocytes was demonstrated in UVB irradiated skin but not in unirradiated skin. Antibody binding to UVB-irradiated keratinocytes in intact epidermis was seen on the cell surface, in the cytoplasm, and in the nucleus. Similar induction of epidermal antibody binding has been reported by Natali and Tan [42] in mice irradiated with UVC and injected with anti-UV-DNA. In more recent experiments, Lee et al [28] demonstrated that antibodies to SS-A/Ro bind on the surface of epidermal keratinocytes in human skin grafted onto nude mice. These mice received injections of anti-SS-A/Ro antisera, and IgG was found to bind to epidermal keratinocytes of engrafted human skin in a pattern similar to that seen in SCLE and NLE skin lesions. Based on these results, we propose that anti-ENA, especially anti-SS-A/Ro and anti-SS-B/La, bind to epidermal keratinocytes in tissue bathed with plasma containing these antibodies. In a certain proportion of cells showing granular cell surface binding of anti-SS-A/Ro, anti-SS-B/La, or anti-RNP, cytoplasmic and nuclear staining also occurs by active internalization. Since this was not seen using anti-DNA reagents, we believe that such internalization is not just passive penetration of antibodies into damaged cells.

It is important to consider the problem of cross-reactive specificities in autoimmune sera [43,44]. We cannot exclude the possibility that antibodies specific for SS-A/Ro or SS-B/La are recognizing other antigens on the cell surface of keratinocytes. However, even if that were the case, the cell-surface binding of antibodies of these specificities from lupus patients to keratinocytes might still be acti-
vators of immunologic cytotoxicity of these targets, with UVL as a trigger factor of antibody binding.

A potential problem with such experiments as these, which use "monospecific" whole antisera as probes, is that cross-reacting antibodies or other antibodies not detected by immunoblotting, CIE, or immunodiffusion might be responsible for the IgG binding to keratinocytes. We tested the possibility in previous experiments on human skin grafted onto nude mice [28,29]. We verified that monospecific anti-SS-A/Ro antibody binding was indeed anti-SS-A/Ro by using affinity purified anti-SS-A/Ro, and by using sera preadsorbed with purified SS-A/Ro antigen. This increased our confidence that we are not just identifying unknown antibody systems. With multiple monospecific anti-SS-A/Ro or anti-SS-B/La specific antisera, or with sera with both specificities, the same staining patterns and augmentation with estradiol [22] or UVL are seen. It is likely that the antibodies that are binding to keratinocytes in these experiments are either SS-A/Ro or SS-B/La specific, because no other antibody specificities were detected by immunoblotting, CIE, or immunodiffusion, and the relative sensitivity of these and the IF techniques used to identify antibody binding are comparable. Also, the verification of specificity in previous studies with affinity purified or specifically depleted antisera supports this conclusion.

We have already shown that cells coated with appropriate ribonucleoprotein antigen can be lysed by monocyte and lymphocyte mediated ADCC in vitro [45]. In the present study, we demonstrate that monospecific anti-ENA (especially anti-SS-A/Ro and anti-SS-B/La antisera) bind with nuclear and cytoplasmic constituents of keratinocytes cultured in a serum-free, low-calcium defined medium and to epidermal keratinocytes from suction-blisters. We also show that cell damage induced by UVL can induce expression of these nuclear and cytoplasmic constituents on the cell surface in a UVB-dependent, glycosylation-dependent, and microfilament-dependent process. We hypothesize that translocation of nuclear antigens to the cell surface in human keratinocytes by UVL plays a pathologic role in making these cells susceptible to the effects of specific antibodies, leading to immunologic keratinocyte lysis by complement activation or more likely by ADCC mediated by lymphocyte or monocyte effectors [46–48].

This work was supported by grant AR26427 from the National Institutes of Health, by Fellowship Awards from the Dermatology Foundation by the Uchera Memorial Foundation, and by grant 01570565 from the Japanese Ministry of Education, Science and Culture.

**Figure 5.** Panels A–D show suction blister roof epidermis from non-irradiated (A) and UVB-irradiated (B–D) segments of the forearm of a human volunteer. After the blister roofs were harvested, the intact epidermis was incubated in serum containing both anti-SS-A/Ro and anti-SS-B/La for 16 h at 4°C. The samples were washed, fixed in cold acetone, embedded in OCT compound, sectioned, and then incubated with fluorescent conjugated anti-human IgG. The final staining was done in the presence of paraphenylenediamine (PPD) to provide nuclear counterstaining. Panel A shows non-irradiated skin. Faint nuclear counterstaining is seen throughout the epidermis. Occasional basal keratinocytes show diffuse fluorescence indicated by black arrowhead, probably due to cell damage during blister formation. Similar non-specific staining was seen using normal human serum probes or second antibody alone. (Magnification ×125.) In panels B–D, three types of fluorescence are seen, indicating anti SS-A/Ro or SS-B/La staining of epidermis: nuclear staining is shown by white arrowhead; granular or diffuse cytoplasmic staining is shown by white arrow; and cell-surface linear or granular staining is shown by black arrow. (Magnification ×125.) Granular or linear cell-surface staining is seen particularly well in some cells in panels B and C. In panel D, diffuse cytoplasmic staining is clearly seen, with sparing of the intercellular space, which appears as a dark boundary between cells. Since antibody probes were added to intact, viable epidermis in short-term organ culture, the subcellular localization indicates antibody penetration or active internalization into viable keratinocytes.
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