Rapid Publication

Ultraviolet Light Induces Binding of Antibodies to Selected Nuclear Antigens on Cultured Human Keratinocytes

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Abstract. Antibodies which bind to different nuclear antigens in tissue sections or in permeabilized cell cultures are useful markers of subsets of connective tissue disease, especially of lupus erythematosus (LE), but whether these antibodies are able to react with these intracellular sequestered antigens in vivo and cause immunologic tissue damage has been a matter of much debate. We report experiments which show that ultraviolet light-irradiated, cultured human keratinocytes bind IgG antibodies from the sera of LE patients with either monospecific anti-SSA/Ro, anti-RNP, or anti-Sm activity, which implies that these antigens have been made accessible on the cell surface by ultraviolet irradiation. Normal human sera or LE patient's sera with anti-double-stranded DNA, anti-single-stranded DNA, or antihistone activity do not bind to the surface of irradiated human keratinocytes. In control experiments, all antisera produced the expected patterns of nuclear and cytoplasmic staining of fixed permeabilized, unirradiated keratinocytes. Careful study of the viability and permeability of irradiated keratinocytes by several techniques showed that this apparent cell membrane expression of extractable nuclear antigens (SSA/Ro, RNP, and Sm) following irradiation was seen on injured keratinocytes whose cell membranes were intact, but not on dead cells. It is particularly significant that all six monospecific anti-SSA/Ro sera bound to irradiated keratinocytes, since this antibody antigen system is highly associated with photosensitive cutaneous LE.

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Introduction

Lupus erythematosus (LE)[1] is a multisystem autoimmune disease with multiple but distinctive clinical subsets. A unifying feature of these LE subsets and of animal models of LE is circulating autoantibodies whose production appears to be related to abnormal control of the immune response (1). Antinuclear antibodies (ANA) and antibodies to specific nuclear antigens are widely used to diagnose LE and to identify specific subsets of disease (2).

Cutaneous lesions are a frequent component of several LE subsets and are frequently associated with ultraviolet light (UVL) exposure (1). Autoantibodies may be related to the pathogenesis of cutaneous LE since deposits of immunoglobulin and complement occur at the dermal-epidermal junction in LE patients, especially in sun-exposed skin (3). These deposits can be induced experimentally by UVL (4), and are associated with the inflammatory infiltrate and with keratinocyte damage seen in cutaneous LE. It has been shown that UVL induces the release of nuclear antigens which bind with specific antibody in the skin (5), but the cellular source of these antigens and the mechanism of release has not been well-studied. Our hypothesis is that even sublethal doses of UVL may affect nuclear antigen distribution within epidermal keratinocytes and lead to antigen expression in locations where these antigens can interact with components of the immune response. We

1. *Abbreviations used in this paper:* ANA, antinuclear antibody; DSDNA, double-stranded DNA; FD, fluorescein diacetate; LE, lupus erythematosus; RNP, ribonucleoprotein; Sm, Smith antigen; SSA, Sjogren syndrome-A; SSDNA, single-stranded DNA; UV, ultraviolet, UVL, ultraviolet light.

used well-characterized sera from LE patients containing specific antibodies to different nuclear antigens as probes to study the expression of nuclear antigens on human keratinocytes in culture following UVL irradiation. Injurious but sublethal doses of UVL stimulated cultured human keratinocytes to selectively express Sjogren syndrome-A (SSA), ribonucleoprotein (RNP), and Smith antigen (Sm) nuclear antigens on their cell membranes. Autoantibodies to SSA are most highly associated with photosensitive skin disease in LE (6–8).

Methods

Irradiation of epidermal cell cultures. Human keratinocytes were isolated from neonatal foreskins in primary culture in low calcium (0.1 mM), serum-free defined medium by the method of Boyce and Ham (9). Under these conditions, nonstratifying discrete monolayer colonies are produced. First passage cultures were maintained in glass chamber slides (Lab Tek, Miles Laboratories, Inc., Elkhart, IN) for irradiation and subsequent immunofluorescence evaluation. Staining with antikeratin antibodies confirmed that 100% of the cells in culture were keratinocytes.

When nonstratified colonies of 10–50 cells had developed, the cultures were irradiated with 0, 0.2, or 2.0 mJ/cm^2 UVL emitted from a Hanovia Luxor hot quartz lamp which is predominantly a UVB source but which also emits UVC and UVA. At 8 and 24 h after irradiation, the cultures were examined for binding of antibody, for viability, or for cell permeability as described below.

The doses of 0.2 and 2.0 mJ/cm^2 were chosen to achieve cell injury without killing all the cells. Since 10% of UVB light incident on Caucasian human skin penetrates to the basal layer, an in vitro dose of 2 mJ/cm^2 would be equivalent to 20 mJ/cm^2 to in vivo human skin. The basal layer is most relevant since it is the epidermal germinative cell layer and it is where most of the epidermal cell damage occurs in LE. One MED in Caucasians ranges from 15–75 mJ/cm^2 for UVB light. Therefore, our in vitro doses are approximately equivalent to one MED or less in vivo.

Immunofluorescent measurement of IgG binding to irradiated keratinocytes. Five sera from LE patients which were "monospecific" in their reactivity to nuclear antigens, as described in Table I, were selected to determine antibody binding to irradiated keratinocytes. Five additional "monospecific" anti-SSA-Ro sera were eventually tested. A mouse monoclonal IgG antibody with affinity for histone was also used. Normal human sera or a mouse hybridoma medium negative control were used as negative controls in all experiments. Experiments examining IgG binding after incubation with each antisera were repeated at least three times with consistent and reproducible results.

Binding of antibodies from monospecific antisera was measured with an improved indirect immunofluorescence technique using propidium iodide to provide nuclear detail (10) and paraphenylenediamine to preserve fluorescence (11). Binding of human IgG was detected by binding of a fluoresceinated rabbit anti-human IgG (Calbiochem-Behring, Corp., San Diego, CA), and binding of the monoclonal antihistone was determined with a fluorescein-conjugated goat F(ab')^2 anti-mouse IgG as the second reagent (Tago, Inc., Burlingame, CA).

Human IgG binding to cell surfaces was measured by first blocking irradiated cultures with normal rabbit serum, and then incubating keratinocytes with a 1:10 dilution of monospecific antiserum followed by acetone fixation, second antibody incubation, and sequential propidium iodide dip and mounting in a paraphenylenediamine-containing mounting medium.

*Table I. Specific Antisera**

Anti-sera	ANA titer	ANA pattern	Antibody specificity RNP	SSA	SSB	Sm	DNA
Anti-RNP	1/256	speckled	1/10^5	—	—	—	—
Anti-Sm	1/256	speckled	—	—	—	1/32,000	—
Anti-SSA(Ro)	1/16	speckled	—	1/32	—	—	—
Anti-SSDNA	1/256	homogenous	—	—	—	—	30–40% binding SSDNA§
Anti-DSDNA‡	1/256	rim & homogenous	—	—	—	—	50% binding DSDNA§

* ANA titer and pattern were determined on mouse kidney substrate using standard indirect immunofluorescence techniques (12). The titer of anti-RNP was determined by passive hemagglutination, and anti-SSA(Ro), anti-Sjogun syndrome-B (SSB), and anti-Sm by immunodiffusion, as previously described (12). The level of anti-DNA (both SSDNA and DSDNA) were determined by a modified Farr technique (13).

‡ Also had antihistone antibody as determined by histone reconstitution (14).

§ Normal level < 5% binding.

To determine the binding of human IgG from monospecific sera to cytoplasmic and nuclear structures, the cells were acetone fixed to permeabilize membranes firstly and, then, blocked with rabbit serum and incubated with the antisera from LE patients followed by second antibody, propidium iodide, and PPD mounting medium. The degree of positive staining was determined qualitatively and quantitatively by visual counting of at least 200 cells in each specimen using a Zeiss epi-illuminated microscope (Carl Zeiss, Inc., Thornwood, NY). The percentage of cells staining and the degree of staining on a 0–4 scale were determined (Table II).

Cell viability measurements. Cell viability in irradiated cultures was measured by ethidium bromide with acridine orange (15) or by propidium iodide exclusion (16). These results were verified by fluorescein diacetate (FD) uptake (15), which requires intact cell membranes and active metabolism of the fluorochrome for visualization.

In a separate series of double-labeling experiments, we simultaneously examined binding of human IgG from LE patients' sera and viability of irradiated keratinocytes in two different ways. In the first technique, FD uptake was observed in nonfixed cell cultures incubated first with monospecific antisera and then with affinity-purified rhodamine-labeled goat antihuman IgG (Tago, Inc.). By visualizing the double labeling, we were able to identify whether the irradiated cells binding IgG from LE patients' sera were metabolically active and had intact cell membranes. In another technique, we took advantage of the observation that cells with damaged membranes allowed the mouse monoclonal antihistone IgG to enter and stain the nucleus. In this double-labeling technique, the monospecific LE antisera and mouse monoclonal antibody were added to nonfixed, irradiated cells, and the human IgG staining was identified with a rhodaminated affinity-purified rabbit anti-human IgG (Calbiochem-Behring Corp.) and the mouse monoclonal was identified by a fluoresceinated affinity-purified anti-mouse IgG (Tago, Inc.). This enabled us to simultaneously identify binding to nuclear antigens on the cell surfaces and to determine whether the cells were permeable to the antihistone or not.

Cycloheximide. Cell cultures were preincubated with cycloheximide

(Sigma Chemical Co., St. Louis, MO) at concentrations of 0, 0.25, 2.5, or 25 μg/ml for 4 h prior to irradiation, were maintained at the same concentrations of cycloheximide for 10 and 24 h after irradiation, and then were examined for binding of human IgG to their cell surfaces after incubation with LE patients' monospecific sera.

Results

Cells irradiated with either 0.2 or 2.0 mJ/cm^2 UVL bound IgG found in LE patients' sera which were monospecific for anti-RNP, anti-Sm, or anti-SSA/Ro, but did not bind IgG from normal human sera, from LE patients' sera containing high titer anti–single-stranded DNA (SSDNA) or anti–double-stranded DNA (DSDNA) or mouse IgG monoclonal antihistone antibody. As summarized in Table II, this effect was seen at both 8 and 24 h, producing 3+ granular fluorescent staining of irradiated cells. Nonirradiated cells carried for 8 or 24 h exhibited only rare, variable granular staining. This selective staining of the extractable nuclear antigens RNP, Sm, and SSA/Ro is shown visually in Fig. 1. In Fig. 1 *A*, irradiated cultures at 0 h show no cell membrane staining with anti-SSA/Ro; nuclei counterstained with propidium iodide appear prominently. In Fig. 1 *B*, dense granular cell membrane staining with anti-SSA/Ro is seen on a group of irradiated keratinocytes at 8 h. Similar staining was seen at both doses of light at 8 or 24 h. The coarse granular staining with anti-SSA was not accompanied by nuclear staining, implying that the antibody probes were binding cell surface antigen and were not entering the cell to bind to nuclear or cytoplasmic determinants. Anti-RNP serum occasionally produced nuclear staining on non-permeabilized cells, implying penetration into living cells as has been previously reported (18) with antisera of this specificity. The granular staining by anti-RNP, anti-Sm, or anti-SSA was infrequently observed without UV irradiation, and granular staining was never seen with normal human serum or with antibodies to DNA or histone.

Table II. Effect of Irradiation on Cell Surface Expression of Nuclear Antigens

Specificity of antisera	0 h*	8 and 24 h*
Anti-RNP	+/−	3+
Anti-Sm	+/−	3+
Anti-SSA/Ro	+/−	3+
Anti-SSDNA	0	0
Anti-DSDNA	0	0
Anti-histone	0	0
Normal human sera	0	0

* The grading reflects fluorescent intensity on a scale of 0–4+. In most experiments, 10–20% of the cells exhibited the granular fluorescence after UV irradiation, but as few as 5% or as many as 50% of the keratinocytes in some experiments showed this granular staining following UV irradiation. If only occasional cells stained (<5%), then a "+/−" designation is used. A grade of "0" means that no cells stained.

Strong granular staining (3+) of unfixed irradiated keratinocytes was seen with all six monospecific and anti-SSA/Ro sera studied. The percentage of positive cells in irradiated cultures usually ranged from 10–20% and, occasionally, individual slide chambers showed even more staining. In unirradiated controls, less than 1% of the keratinocytes showed staining of any intensity, even faint granular staining. Keratinocytes permeabilized with acetone consistently showed nuclear and cytoplasmic staining with anti-SSA/Ro in 100% of keratinocytes with all anti-SSA/Ro sera tested.

If cell membranes of cultured keratinocytes were made permeable by acetone treatment before incubation with the LE antisera, nuclear staining patterns were seen that were appropriate for the serum specificities (as described in Table I), and were seen with all antisera. Prior UV irradiation of keratinocytes accentuated the SSA- and RNP-speckled nuclear fluorescence seen in permeabilized cultures.

Since the granular staining was seen only with antibodies to certain antigens, and was associated with accentuated nuclear staining by antibodies to those same antigens, we felt that this phenomenon was not the result of cell death. We initially examined the viability of our irradiated cell preparations in two ways: ethidium bromide with acridine orange (15) and propidium iodide exclusion (16). Both viability assays gave similar results. With no irradiation, cells harvested at 24 h were ~80% viable after all the incubations. With 0.2 or 2.0 mJ/cm^2 UVB irradiation, the keratinocyte viability at 24 h was 55 and 46%, respectively. The degree of cell death with irradiation was similar to that reported in other UV-irradiated keratinocyte experiments (17).

Simultaneous assessment of viability by FD uptake and of cell surface binding of anti-SSA or anti-RNP counterstained with rhodamine-labeled second antibody was done to determine whether the irradiated cells expressing cell surface SSA/Ro or RNP were metabolically active. We found that irradiated keratinocytes which showed granular staining with anti-SSA or with anti-RNP also stained positively with FD, but less intensely than did cells without granular staining. In Fig. 2 *A*, the fine granular staining is rhodamine-labeled binding to anti-SSA, while the globular or diffuse stain is FD uptake. This demonstrated that these cells were viable but apparently injured.

Simultaneous assessment of cell membrane permeability by internalization of mouse monoclonal antihistone antibody counterstained with fluoresceinated second reagent, and of cell surface anti-SSA or anti-RNP counterstained with rhodamine-labeled antibody were also done. Viable, unirradiated keratinocytes did not show cytoplasmic or nuclear staining with this antihistone unless their membranes were made permeable by acetone fixation. As seen in Fig. 2 *B*, irradiated keratinocytes showing granular anti-SSA staining did not stain with the monoclonal antihistone antibody. The permeable cells with dense antihistone staining did not show rhodamine positive granules, indicating that anti-SSA binding did not occur on

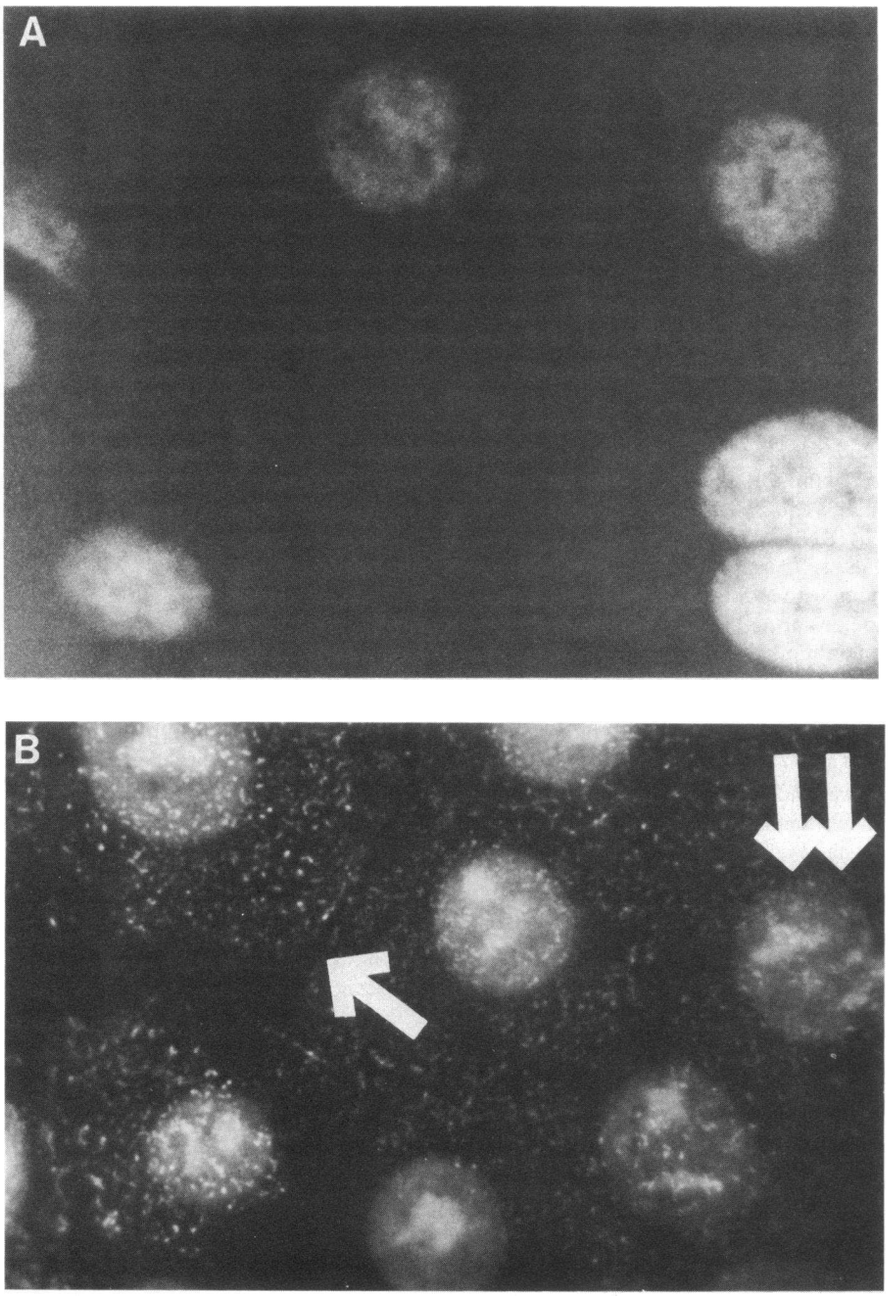

Figure 1. (*A*) Staining with anti-SSA/Ro of irradiated cells at 0 h post irradiation. Only nuclei counterstained with propidium iodide are seen. (*B*) Granular cell membrane staining with anti-SSA/Ro serum as seen at either 8 or 24 h post UVL. Note that the entire cell membrane is stained in a granular manner with visible black spaces between cells (single arrow). Nuclei are counterstained with propidium iodide for detail (double arrow). Granular anti-SSA binding was detected with a green fluorochrome, while propidium iodide nuclear staining was red.

the cell surface in these permeable cells. With anti-RNP antisera, granular cell surface staining was only occasionally seen in cells permeable to the antihistone monoclonal antibody, but speckled nuclear staining was also seen in these cells. Anti-Sm sera were not tested in this system. The demonstration that the vast majority of cells with granular staining have intact cell membranes further suggests that the granular staining represents cell membrane antigen expression post-UVL irradiation.

Using these two assays, three cell populations were demonstrated post-UV irradiation (Fig. 3). First, there was a viable uninjured ("live") population comprising 25–35% of the cells which avidly hydrolyzed and retained FD, and was impermeable to the monoclonal antihistone antibody. A second viable

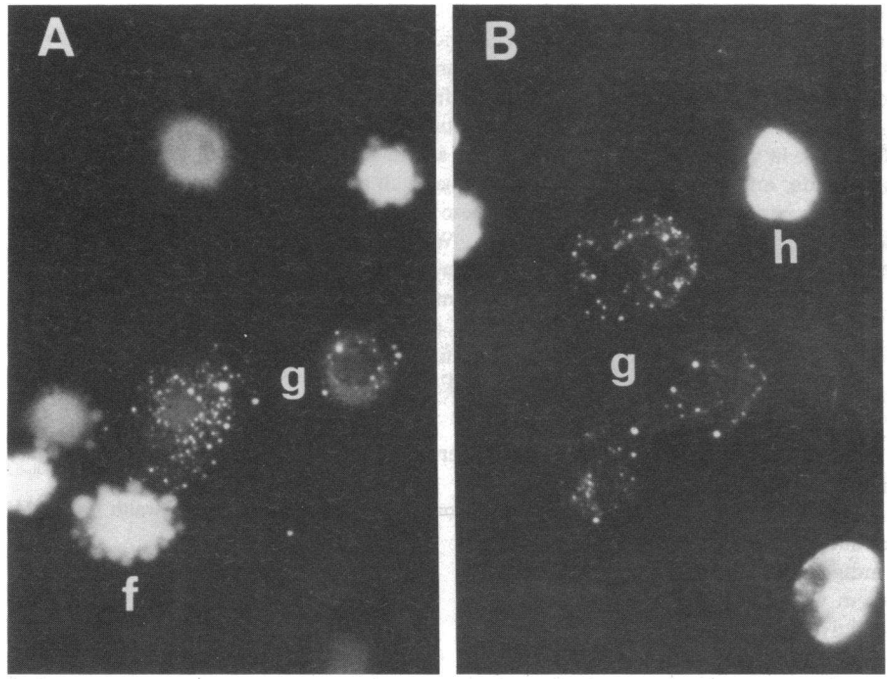

Figure 2. Metabolic studies of cells with granular anti-SSA and anti-RNP cell membrane staining. (*A*) Cells with granular anti-SSA cell membrane staining (*g*) stain less brightly with FD than do the uninjured cells (*f*-diffuse white cells in this photograph). (*B*) Cells with granular anti-SSA staining (*g*) are not permeable to the monoclonal antihistone antibody which gives a bright nuclear and perinuclear stain (*h*). Cells with granular anti-SSA or RNP staining (*g*) were identified by a rhodaminated second antibody showing red fluorescence, while cells labeled *f* or *h* showed green fluorescence due either to FD (*f*) or fluoresceinated second antibody (*h*). No propidium iodide nulear stain was used in these experiments.

"injured" population comprising 10–20% of the cells was seen which hydrolyzed and retained FD poorly, but remained impermeable to the monoclonal antihistone antibody. This is the population which had the granular staining with anti-SSA and anti-RNP post UVL. Because these cells were impermeable to antibody, the granular staining seen was likely due to antigens on the cell membrane. There were occasional cells with nuclear staining by the anti-RNP antisera in this group; however, the majority of irradiated cells which stained with anti-RNP had granular but not nuclear staining. This is consistent with a previous report of viable keratinocytes internalizing anti-RNP antibodies (18). A third population of nonviable ("dead") cells comprising 45–55% of the cells was consistently seen. These cells did not hydrolyze and retain FD, and were permeable to the monoclonal antihistone antibody. This population had no cell membrane staining with the anti-SSA serum and showed occasional granular staining with anti-RNP (<1%), but did have frequent nuclear staining patterns with the anti-RNP sera. This degree of cell death is similar to that observed in previous experiments using in vitro irradiation of keratinocytes (17). We interpret these results as evidence that UV irradiation of keratinocytes in culture causes cell death and irreversible membrane damage to many cells, but also produces a population of injured cells with expression of certain nuclear antigens on the cell membranes.

This cell membrane expression of RNP and SSA antigens

	Live	Injured	Dead
Fluorescein diacetate metabolism & retention	+	±	-
Permeable to monoclonal antibody	-	-	+
Cell membrane staining with LE antisera	-	+	occasionally + RNP only
Permeable to LE antisera	-	occasionally + RNP only	+ RNP only

Figure 3. Three cell populations.

was almost completely blocked both quantitatively and qualitatively by inhibiting protein synthesis in the cultures with cycloheximide shortly before and after irradiation. This blocking increased with increasing doses of cycloheximide from 2.5 to 25 μg/ml. Assessment of cell viability by ethidium bromide with acridine orange showed no significant decrease in viability of irradiated cells with any dose of cycloheximide, and total cell numbers were not apparently affected by these conditions. Thus, cell surface expression of these nuclear antigens appears to be active, and is protein synthesis-dependent.

Discussion

We have demonstrated that antigens normally detected in the nucleus and cytoplasm of normal human keratinocytes can be induced to appear on the cell surface of keratinocytes under conditions of ultraviolet light-induced cell injury, using binding with antisera from patients with LE as indicators of specific antigen expression. This apparent cell surface antigen expression was selective for the extractable nuclear antigens RNP, Sm, and SSA/Ro, but did not occur in our system with DSDNA, SSDNA, or histones. We feel that this is an active process, but it is also possible that the binding of anti-SSA, RNP, and Sm antibodies to cell membranes was due to passive leakage of antigen in dying cells. We feel that the latter possibility was unlikely because the phenomenon was dependent on the production of sublethal injury and was seen in cells without altered membrane permeability, the expression was selective, and active protein synthesis appeared to be required for this phenomenon to occur.

There has been much debate whether antibodies to sequestered nuclear constituents are pathogenetically involved in the production of tissue damage other than nephritis in LE. We have demonstrated that selected nuclear antigens may be detected on the surface of keratinocytes following UVL-induced damage, where these antigens are available to bind antibodies from LE patients' sera. This is particularly pertinent in photosensitive cutaneous LE where keratinocytes are indeed exposed to significant UVL and where keratinocyte damage is a significant histologic characteristic. In addition, release of these antigens from keratinocytes following UVL damage may lead to deposition of antigen antibody complexes at the dermal-epidermal junction, and to systemic antigen release, as has been suggested by previous experiments in mice (4–5).

The demonstration of SSA/Ro antigen in the nucleus and cytoplasm of viable human epidermal keratinocytes and on the cell surface of UVL-damaged keratinocytes is of particular concern to those interested in the pathogenesis of photosensitive cutaneous LE. This antibody system has been highly associated with three photosensitive cutaneous LE syndromes: neonatal LE (6), subacute cutaneous LE (7), and ANA negative LE (8). Although the antigen has been identified in spleen, liver, and kidney sections (19), in peripheral blood lymphoid cells, and in epithelial and B lymphocyte cell culture lines (20, 21), there has been some difficulty in identifying it in intact human skin (22). In these experiments, we show that all six monospecific SSA/Ro antisera from LE patients bind with nuclear and cytoplasmic constituents of cultured nonstratified human keratinocytes using a serum-free, low calcium-defined medium, and that cell damage such as that induced by UVL can induce expression of these nuclear and cytoplasmic constituents on the cell surface. We postulate that modulation of nuclear antigen localization in human keratinocytes by UVL or other factors is important in making these cells susceptible to the effects of specific antibodies, such as anti-SSA, leading to immunologic keratinocyte destruction as is seen in photosensitive cutaneous LE.

Acknowledgments

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