Study of HLA-DR Synthesis in Cultured Human Keratinocytes*

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Within the normal human epidermis only Langerhans and indeterminate cells express HLA-DR. Human keratinocytes (HK), however, may also express HLA-DR in certain disease states characterized by mononuclear cell infiltrates. Previous studies have shown that HK synthesize HLA-DR in response to stimulation by interferon gamma (INF-γ). The purposes of this study were to define conditions under which cultured HK might express HLA-DR and to compare the HLA-DR synthesis of HK with that of monocytes. HLA-DR expression by HK as determined by indirect immunofluorescence of HK cultures was absent under standard low calcium conditions and remained absent with the addition of calcium, serum, mitogens, and supernatants from Pam-212 cells containing epidermal thymocyte-activating factor. HLA-DR expression in HK was induced by cocultivation with concanavalin A-stimulated peripheral blood mononuclear cells (PBMC), but not unstimulated PBMC. This effect was time-dependent and directly related to the number of PBMC. HLA-DR expression was also induced in a time- and dose-dependent manner by addition of supernatant from stimulated PBMC (SS) or by addition of recombinant INF-γ but not by addition of interleukin (IL)-1 or IL-2. Induction by either SS or INF-γ was blocked by an antiserum to INF-γ. As determined by a semiquantitative immunoprecipitation technique, HLA-DR synthesis by HK was directly related to INF-γ concentration. The pattern of HLA-DR peptides produced by HK was similar to that of monocytes, but the relative quantity synthesized was far less than that of monocytes. *J Invest Dermatol 87:559–564, 1986*

Within the normal human epidermis, expression of class II or HLA-DR antigens is confined to the dendritic Langerhans cell [1]. However, in certain disease states, such as graft-versus-host disease, lichen planus, mycosis fungoides, and skin-graft rejection, the keratinocytes of the epidermis may also express HLA-DR antigens on their surfaces [2–6]. The disease entities associated with HLA-DR expression by keratinocytes are ones characterized histologically by inflammatory infiltrates of mononuclear cells in the skin and are thought to involve cell-mediated immune reactions in the skin.

Our understanding of the phenomenon of HLA-DR expression by cells has advanced considerably in recent years. Several groups [7–10] have clearly demonstrated that HLA-DR expression by keratinocytes is due to the synthesis of these proteins by keratinocytes and not to passive acquisition of the proteins from other cells. Other studies [11–13] have demonstrated that the signal for induction of HLA-DR synthesis by keratinocytes is gamma interferon (IFN-γ).

In an attempt to better understand HLA-DR expression by keratinocytes, we have studied HLA-DR expression in cultures of human keratinocytes, attempting to stimulate in vitro the conditions that lead to HLA-DR expression in vivo. We have confirmed the role of IFN-γ in induction of HLA-DR expression and have also demonstrated that the HLA-DR peptides synthesized by keratinocytes are qualitatively similar to but quantitatively less than those of monocytes.

MATERIALS AND METHODS

Human Keratinocyte Cultures Human keratinocytes (HK) were isolated from neonatal foreskins and grown in a 0.1 mM calcium, serum-free medium 153, as described by Boyce and Ham

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Abbreviations:
AM: adherent monocytes
BSA: bovine serum albumin
Con-A: concanavalin A
cpm: counts per minute
DOC: sodium deoxycholate
ETAF: epidermal thymocyte-activating factor
FCS: fetal calf serum
HK: human keratinocytes
IFN-γ: interferon gamma
IIF: indirect immunofluorescence
IL-1: interleukin 1
IL-2: interleukin 2
NRS: normal (preimmune) rabbit serum
PBS: phosphate-buffered saline
PHA: phytohemagglutinin
PBMC: peripheral blood mononuclear cells
SDS: sodium dodecyl sulfate
SPA: staphylococcal protein A
SS: supernatants from stimulated PBMC
TCA: trichloroacetic acid

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[14]. For these studies, second-passage keratinocytes were grown on 2-chamber Lab-Tek slides. By indirect immunofluorescence staining for keratins, all cells in culture on the slides were keratinocytes.

Indirect Immunofluorescence Examination of HK for HLA-DR was performed by indirect immunofluorescence (IIF). Cells on slides were washed 3 times in phosphate-buffered saline (PBS) (10 mM Pi, 150 mM NaCl, pH 7.4) fixed and permeabilized in cold (−20°C) acetone, and air-dried. Cells fixed on slides were incubated for 16 h at 4°C with a 1:100 dilution of a murine monoclonal antibody in PBS with 0.1% sodium azide and 5% normal goat serum. Slides were then washed in PBS, incubated 2 h in a 1:100 dilution of goat antimouse IgG F(ab')2 (Tago Inc., Burlingame, California), and washed again in PBS. Coverslips were mounted over a glycerol-PBS mounting medium (pH 8.5) containing 0.1% paraphenylenediamine [15]. Slides were viewed under an Olympus epifluorescence microscope equipped with an HBO-100 mercury light source and a filter-reflector system for fluorescein. The percentage of HK staining positively for HLA-DR on each half of a slide was derived by counting positive and negative cells (at least 200) in 10 high-power (40×) fields.

Antibodies and Antisera As positive control antibodies for identification of HLA antigens on HK in culture, murine monoclonal antibodies to HLA-A,B,C (Clone MB40.5, Atlantic Antibodies, Scarborough, Maine) and to β_2 -microglobulin (Clone L368, Becton-Dickinson, Sunnyvale, California) were used. A monoclonal antibody, OKT6, was used as a negative control and for verification that Langerhans cells were absent from the cultured epidermal cells. For identification of HLA-DR by IIF, an IgG_1 monoclonal antibody (Clone MCD1/2C3, Atlantic Antibodies) was used. For immunoprecipitation of HLA-DR, an IgG_{2a} monoclonal antibody (Clone L243, Becton-Dickinson) was employed. Rabbit antiserum to IFN-γ was from Interferon Sciences, New Brunswick, New Jersey.

Peripheral Blood Mononuclear Cells Peripheral blood mononuclear cells (PBMC) from normal donors were isolated over Ficoll-Hypaque [16]. Stimulated supernatants (SS) from PBMC were obtained by culture of PBMC for 3 days in MCDB 153 in the presence of 10% fetal calf serum (FCS) and 10 μg/ml concanavalin A (Con-A).

Pam-212 Cells Pam-212 is the designation for a transformed mouse epidermal cell line [17]. These cells were generously provided by Dr. Stuart Yuspa of the National Cancer Institute. They were grown in serial culture in minimal essential medium (GIBCO, Grand Island, New York) plus 8–10% Hyclone-screened FCS (Hyclone, Logan, Utah). Conditioned medium from these cultures was used as a source of epidermal thymocyte-activating factor (ETAF).

Stimulation of HLA-DR Expression by HK After 5–7 days in culture on Lab-Tek slides, changes in culture conditions and addition of various stimuli were performed. HLA-DR expression by HK was examined 0–5 days later by IIF. Changes in culture conditions examined included increases in calcium concentrations (up to 0.1 mM), addition of 10% normal human serum, plasma, or FCS, addition of 10 μg/ml Con-A or phytohemagglutinin (PHA), and addition of 10% supernatant from cultured Pam-212 cells containing ETAF. PBMC were added and cocultured with HK along with 10% FCS in the presence or absence of mitogen (10 μg/ml Con-A). Other stimulants added included the SS from PBMC, interleukin 1 (IL-1), interleukin 2 (IL-2; Collaborative Research Inc., Lexington, Massachusetts), and recombinant IFN-γ (Genentech, Inc., San Francisco, California). Blocking of the activity of IFN-γ was achieved by incubating SS or IFN-γ with either a rabbit antiserum to human IFN-γ (10^4 nu/ml, Interferon Sciences, New Brunswick, New Jersey) or normal rabbit serum (NRS). After incubation for 1 h at 37°C, the mixture was added to cultured HK.

Immunoprecipitation of Human HLA-DR Antigens Second-passage HK which had grown in 60-mm plastic dishes to 80% confluence (approximately 10^6 cells/dish) were stimulated for 48 h with 0–100 units/ml IFN-γ. As positive control cells for HLA-DR expression, adherent human monocytes (AM) were obtained by incubation of PBMC in plastic dishes containing RPMI culture medium and 10% fresh human serum [16]. The AM were stimulated with 50 units/ml IFN-γ and were processed by the immunoprecipitation technique in a manner identical to the HK. Methionine-deficient media were added to cultured cells for 4 h. Each plate then received a 2 h pulse of 50 mCi [^{35}S]-methionine. The plates were washed 6 times in cold PBS, and the cells in each plate were lysed at 4°C in 0.5 ml of a PBS buffer containing 0.5% Triton X-100, 0.25% sodium deoxycholate (DOC), 2 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA. Each plate was washed with an additional 0.3 ml of this lysis buffer and was scraped with a rubber policeman. The volume of lysate from each plate was made 1.3 ml with addition of lysis buffer. The lysate was spun at 10,000 g for 15 min, and 0.3 ml of the supernatant was removed for precipitation with trichloroacetic acid (TCA). The remainder was used for immunoprecipitation.

To each 0.3-aliquot of cell lysate supernatant, TCA was added to a final concentration of 10%. After an overnight incubation at 4°C, each sample was centrifuged at 10,000 g for 15 min. Pellets were resuspended in 1 ml of 0.6 N NaOH containing 0.1% sodium dodecyl sulfate (SDS). Samples were placed in Beckman Ready-Solv 566436, and radioactivity was measured in a Beckman model LS-7500 β-counter. The counts per minute (cpm) were corrected for any dilutions in order to reflect the cpm in the original lysate.

Immunoprecipitation was performed according to published procedures [18]. A suspension of fixed Cowan strain *Staphylococcus aureus* was prepared according to the manufacturer's instructions and used as a solid phase source of staphylococcal Protein A (SPA) (Enzyme Center, Malden, Massachusetts). In order to clear from each lysate any proteins that might bind nonspecifically to SPA, each sample was incubated for 1 h with 200 μl of SPA. Samples were centrifuged for 5 min, and the supernatant was removed. SDS was added to the supernatant to a final concentration of 1%. To each sample 10 μl of the undiluted monoclonal antibody to HLA-DR was added, and samples were incubated overnight at 4°C. SPA (100 μl) was added to each sample. After 1 h, the SPA was pelleted and washed once with PBS containing 1% SDS, 1% Triton X-100, 0.5% DOC, and 0.5% bovine serum albumin (BSA). Five additional washes were performed with the same wash solution without BSA. Immune complexes were eluted from the SPA by boiling for 5 min in 20 μl of Laemmli sample buffer. Samples were centrifuged, and the supernatants were applied to the wells of a 5–20% gradient polyacrylamide slab gel containing 1% SDS and with a 5% stacking gel. The gel electrophoresis was performed according to standard methods [19]. After electrophoresis, the separated proteins were electroblotted onto nitrocellulose paper (Trans-blot, Biorad). Autoradiography was performed by exposure of Kodak X-Omat R film (Eastman Kodak, Rochester, New York) to the nitrocellulose paper for 4 days.

The bands on the autoradiograph corresponding to HLA-DR peptides were identified by comparison to prestained molecular weight standards on the nitrocellulose paper and to the bands produced by the positive control cells (human AM). The relative densities of the bands on the x-ray film were measured for each sample with a Zeineh Model SL-2D Scanning Laser Densometer (Bio Med Instruments, Fullerton, California). The densities of the bands were integrated (Apple Computer Co., Cupertino, California) and expressed as arbitrary units for each sample. In order to relate the relative amounts of HLA-DR synthesis to total protein synthesis, an HLA-DR synthesis index, the ratio of the HLA-DR band integral to the TCA-precipitable counts, was used to compare relative HLA-DR synthesis.

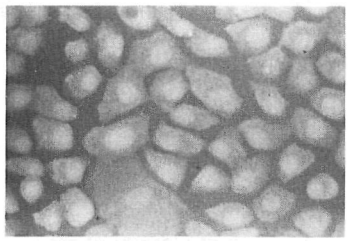

Figure 1. Indirect immunofluorescence of cultured HK for HLA-DR. HK cultured up to 2 weeks and with the addition of calcium, serum, mitogen, and supernatants with Pam-212 cells remained negative for HLA-DR.

RESULTS

Second-passage HK stained positively for HLA-A,B,C and β_2-microglobulin but did not express HLA-DR or OKT6 after culture up to 2 weeks (Fig 1). No HLA-DR expression by HK was noted with changes in calcium concentration, addition of FCS, normal human serum, plasma, mitogens, or ETAF.

When PBMC were added to HK and cocultured in the presence of Con-A (10 μg/ml) for 3 days, HLA-DR expression by HK was seen (Fig 2). The positive staining by IIF was a granular fluorescence that appeared to be accentuated in the perinuclear region rather than just on the periphery of cells. This is the same pattern of fluorescence that was seen when fixed permeabilized HK were stained for HLA A,B,C and β_2-microglobulin. Although HLA-DR$^+$ adherent PBMC were also seen in clusters on the slides, these intensely fluorescent cells were much smaller than HK and were easily distinguished from the keratinocytes.

The relationship of numbers of PBMC and mitogen to HLA-DR expression by HK is shown in Fig. 3. When the ratio of added PBMC to HK in each chamber was 50:1 or less, the percentage of HK expressing HLA-DR after 3 days of cocultivation was no more than 2%. With higher cell ratios, HLA-DR expression by HK increased progressively as did the intensity of the fluorescence. At cell ratios greater than 500:1, HK were destroyed during the cocultivation procedure. Mitogen was an essential element of the induction of HLA-DR in HK: without Con-A, no induction was noted.

The time course of HLA-DR induction is shown in Fig 4. The percentage of HK expressing HLA-DR and the intensity of the staining were highest between 2 and 4 days of cocultivation with mitogen-stimulated PBMC. Beyond 4 days the majority of the HK had been destroyed.

When the supernatant from PBMC stimulated with Con-A for 3 days was added at a 1:1 ratio to the HK culture medium, HLA-DR expression was noted in 70–80% of the HK. This finding implied that a mediator of HLA-DR expression was produced by the stimulated PBMC. Three mediators produced in such cultures, IL-1, IL-2, and IFN-γ, were tested for their ability to stimulate HLA-DR. Neither IL-1 nor IL-2 (50 units/ml) produced HLA-DR expression by HK, whereas recombinant IFN-γ produced HLA-DR expression in a time- and dose-dependent fashion (Fig 5). Little expression was induced at IFN-γ concentrations of 100 units/ml or less, but with higher concentrations, expression was noted within 24 h and approached 80% by 96 h.

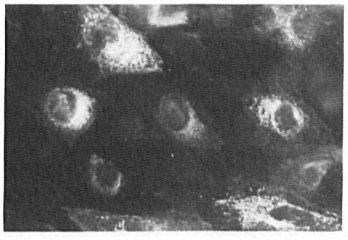

Figure 2. Indirect immunofluorescence of HK cocultured with Con-A stimulated PBMC. The granular staining of permeabilized HK with a perinuclear accentuation represents specific staining for HLA-DR. Permeabilization of the cells does not allow evaluation of their surface antigens.

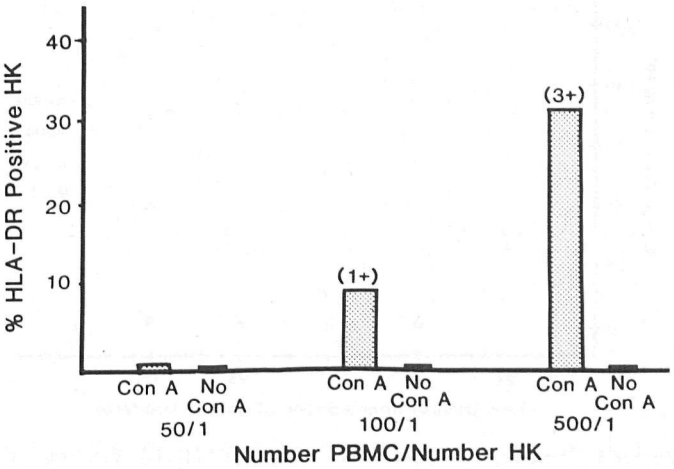

Figure 3. Effect of mitogen-stimulated PBMC on HLA-DR expression by HK. HK were cocultured for 3 days with varying numbers of PBMC in the presence of 10% FCS and either 10 μg/ml Con-A (*shaded*) or no Con-A (*solid bars*). The ratio of PBMC to HK is given on the *abscissa*. The percent of HK staining positively for HLA-DR is given on the *ordinate*. For those conditions in which HK stained positively, the intensity of staining (on a scale of 0 to 3+) is given *in parentheses*.

In order to evaluate whether IFN-γ was the critical mediator of HLA-DR expression, 2 potent stimulators, 500 units/ml of IFN-γ and the SS, were incubated with rabbit antiserum to IFN-γ (10^3 neutralizing units) or NRS and then added to the cultures for 3 days. The induction of HLA-DR in HK by IFN-γ and by the SS was blocked completely by the antiserum to IFN but not by normal rabbit serum.

The results of immunoprecipitation and autoradiography are shown in Fig 6. Monocyte lysates produced a characteristic pattern of bands clustered between M_r 27,000 and 36,000 (lane G). The specificity of this pattern was demonstrated by immunoprecipitation of adherent monocyte lysates with an impertinent murine monoclonal antibody to lysozyme. Under these circumstances, no bands in the M_r 27,000–36,000 K range were produced (Fig 7).

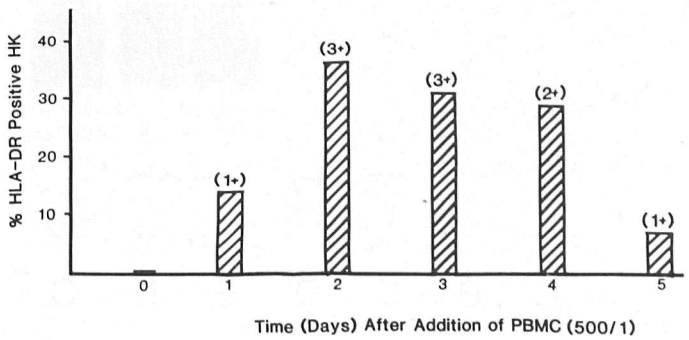

Figure 4. Time course of induction in HK of HLA-DR expression by cocultured monocytes. PBMC were added to HK cultures at a PBMC to HK ratio of 500:1. The cells were then cocultured for 0–5 days. The percent of HK staining for HLA-DR is shown by the *vertical bars*. The intensity of this staining is given *in parentheses* on a scale of 0 to 3+.

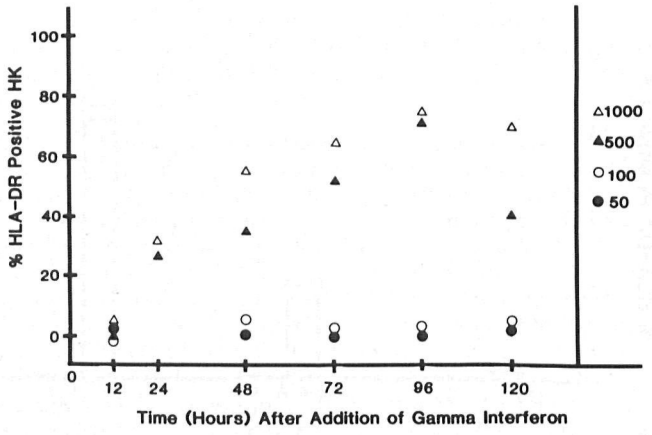

Figure 5. Time and IFN-γ dose dependence of HK HLA-DR expression. IFN-γ, in the concentrations shown (*right panel*, units/ml), was added to cultures of HK. After incubation for 12–120 h, the percentage of positively staining HK was measured and plotted for each concentration and time point.

Immunoprecipitation of HK lysates by anti-HLA-DR produced no such bands if the HK had not been stimulated with IFN-γ (lane A), or had been stimulated with concentrations of IFN-γ less than 50 units/ml (lanes B–D). The lack of the characteristic pattern produced by monocytes confirms that HLA-DR synthesis by normal HK is minimal to absent. When HK were stimulated with IFN-γ at concentrations of 50 or 100 units/ml, bands of HLA-DR peptides similar to those of monocytes were seen (lanes E and F). The lower threshold of IFN-γ concentrations needed for HLA-DR synthesis as detected by this method likely reflects the greater sensitivity of the method compared with immunofluorescence.

The similarity of the peptide bands immunoprecipitated from HK and monocytes is shown by comparison of the densitometer scans (Fig 8). Although not absolutely identical, the major peaks

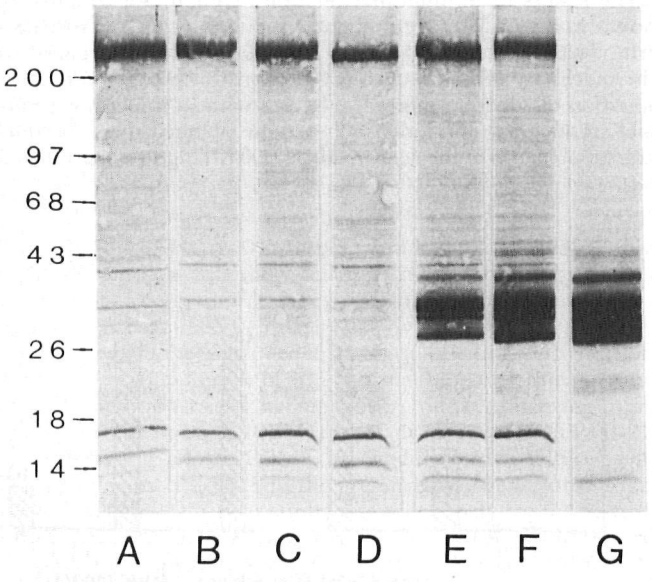

Figure 6. Autoradiograph generated as described in *Materials and Methods* representing proteins immunoprecipitated by monoclonal anti-HLA-DR from lysates of ^{35}S-labeled cells. *Lanes A–F*, keratinocytes. *Lane G*, adherent monocytes. INF-γ doses for lanes A–G were 0, 1, 5, 10, 50, 100, and 50 units/ml, respectively. Migration positions of molecular weight markers (in kD) are shown (*left*).

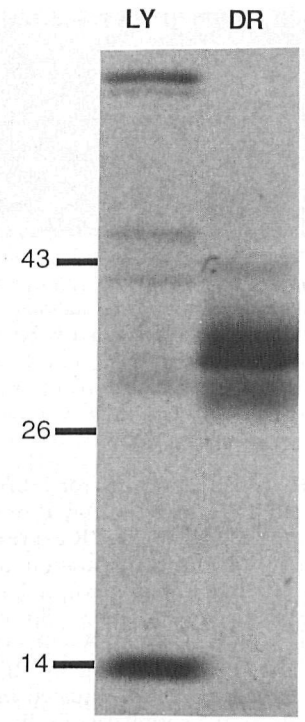

Figure 7. Expanded view of an autoradiograph representing proteins immunoprecipitated from lysates of ^{35}S-labeled adherent monocytes by monoclonal antibodies to lysozyme (*LY*) or HLA-DR (*DR*). Migration positions of molecular weight markers (in kD) are shown (*left*).

are present for both cell types. Quantitation of the immunoprecipitation data is shown in Table I, which presents the IFN-γ concentration, the integral of the densitometer tracings, and the TCA-precipitable counts corresponding to each lane of Fig 6. Only background density is present in HK stimulated with IFN-γ at 10 units/ml or less. A comparison of HLA-DR synthesis

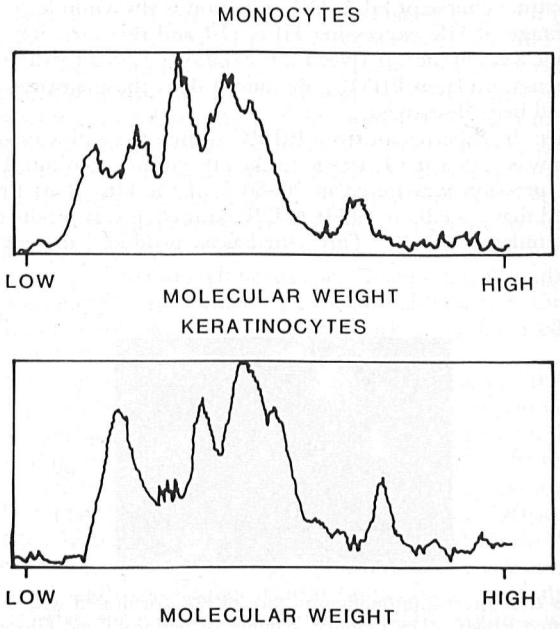

Figure 8. Densitometer scans of lanes G (*upper scan*) and F (*lower scan*) from Fig. 6. The *abscissa* is the scan position along the autoradiograph. The *ordinate* is the band density.

Table I. Stimulation of HLA-DR Synthesis in Human Keratinocytes (HK) and Adherent Monocytes (AM)

	Cell Type						
	HK						AM
IFN-γ (units/ml)[a]	0.0	1.0	5.0	10.0	50.0	100.0	50.0
DR band density[b]	1.5	1.1	1.2	1.3	11.2	18.4	17.5
TCA-precipitable counts[c]	24.0	12.8	20.6	11.5	28.6	16.0	2.6
Ratio[d]	0.9	1.3	0.9	1.7	5.8	17.1	100.0

[a]γ-Interferon concentration.
[b]Band density in arbitrary units.
[c]Expressed as millions of cpm.
[d]Ratio of band density to TCA-precipitable counts expressed as a percentage of the value for AM.

indices is presented in Fig 9. The dose-dependent relationship of HK HLA-DR synthesis to IFN-γ concentration is again evident. However, compared with total protein synthesis (total TCA-precipitable counts), HLA-DR synthesis by stimulated HK is only 17% that of the stimulated monocytes.

DISCUSSION

The initial approach that was taken in these studies of HLA-DR expression by keratinocytes was to attempt to simulate in vitro, the conditions that are associated with this finding in vivo. The disease conditions or experimental situations that are characterized by HLA-DR expression are those which have mononuclear cell inflammatory infiltrates in the skin, and which are thought to involve cell-mediated immune reactions. After determining that alterations in cultured conditions of HK, such as addition of mitogen or serum, did not induce HLA-DR, we cocultured PBMC with HK. Our in vitro finding that cocultivation of PBMC, along with mitogen, led to HLA-DR expression by HK, agrees with the in vivo observation that HLA-DR expression by HK occurs during cell-mediated immune reactions involving monocyte-macrophages and stimulated T lymphocytes.

Our data are consistent with those of Basham et al [11] and Volc-Platzer et al [13] regarding the role of IFN-γ in this phenomenon. The induction of HLA-DR expression by supernatants from stimulated PBMC and by recombinant IFN-γ, the blocking of induction by both stimuli with an antibody to IFN-γ, and the dose–response relationship of HLA-DR expression to IFN-γ concentrations, confirm that IFN-γ is the mediator produced in these systems that leads to HLA-DR expression by other cells. Other mediators, such as ETAF, IL-1, and IL-2 (alone or in combination) were ineffective in this regard.

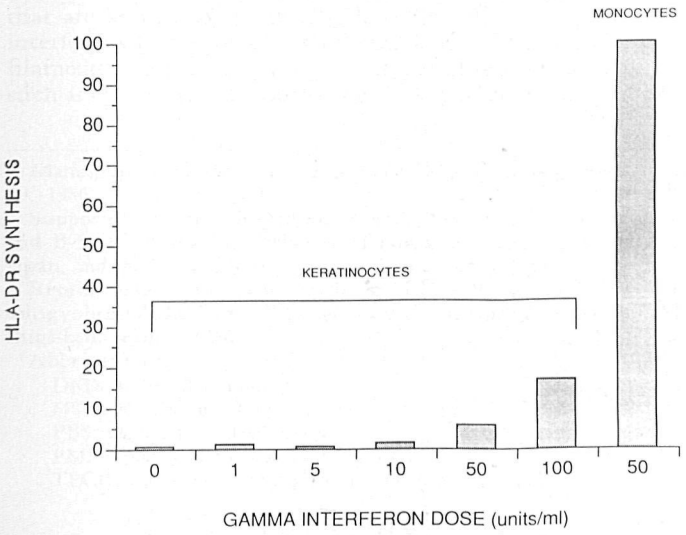

Figure 9. The ratio of HLA-DR band density to total protein synthesis (as measured by TCA-precipitable counts) is expressed in arbitrary units on the *ordinate*. Cell types and IFN-γ concentrations are as shown.

The results of these studies also confirm by direct analysis that HLA-DR expression by keratinocytes is due to de novo synthesis of HLA-DR peptides, rather than to passive acquisition of the antigens from other cells. The appearance, in fixed, permeabilized HK, of HLA-DR staining predominantly within the cells and accentuated in the perinuclear region implies synthesis within the cells (Fig 2). Furthermore, we were able to induce HLA-DR expression in HK easily with SS and IFN-γ without addition of any known HLA-DR$^+$ cells. Finally, the incorporation of [^{35}S]-methionine into peptides of the appropriate molecular weight immunoprecipitated by monoclonal antibody to an HLA-DR common determinant is the conclusive demonstration that the peptides of HLA-DR are synthesized by keratinocytes under appropriate stimulatory conditions.

According to our analysis of the peptides immunoprecipitated from IFN-γ-stimulated HK, the pattern of peptides is similar to, if not identical to, that of monocytes. This is consistent with the work of Morhenn et al. [10] showing that HLA-DR molecules synthesized by keratinocytes have the same charge and molecular weight as those synthesized by autologous lymphocytes. By IIF staining, HLA-DR$^+$ HK are never as strongly positive as monocytes. The results of our attempts to quantitate HLA-DR production by HK shows that monocytes stimulated with half of the IFN-γ concentration as HK, produce 5–10 times as much HLA-DR peptides.

Even though the phenomenon of HLA-DR expression by keratinocytes is relatively well understood at this point, the role of HLA-DR on keratinocytes is still unknown. Whether HLA-DR$^+$ HK serve a role in interaction with the immune system has not yet been clarified. A postulated role for keratinocytes as antigen-presenting cells has not been verified. In fact, it is not yet clear that HLA-DR$^+$ HK may stimulate an allogeneic lymphocyte response. Studies by Roberts et al. [20] have suggested that one possible role of the HLA-DR$^+$ epidermis is to facilitate the movement of the Langerhans cell, an antigen-presenting, bone marrow-derived dendritic cell, into the epidermis. One possibility is that expression of these histocompatibility antigens by HK in disease states or experimental conditions is an epiphenomenon that simply serves as a convenient marker of a stimulated cell-mediated immune reaction, and IFN-γ production. Now that HLA-DR expression can be reliably induced in HK by IFN-γ, and now that recombinant IFN-γ is readily available, it should be possible to understand whether the phenomenon of HLA-DR expression by HK is important in epidermal–immune system interactions.

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