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Induction of the Synthesis of Triton-Soluble Proteins in Human Keratinocytes by Gamma Interferon

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Recombinant human gamma interferon (r-IFN-γ) induces the synthesis and expression of HLA-DR antigen on cultured, normal, human keratinocytes depleted of Langerhans cells. After removal of r-IFN-γ from the culture medium of keratinocytes that are expressing HLA-DR antigen, the cells continue to express this antigen for at least 2 days. r-IFN-γ induces, in a dose dependent fashion, the synthesis of several triton-soluble proteins with the most prominent having an apparent molecular weight of 53,000.

Whereas normal keratinocytes do not express HLA-DR antigen in vivo, they do express HLA-DR in a variety of skin diseases such as lichen planus, graft-versus-host disease, and mycosis fungoides. We propose that an understanding of lymphocyte-keratinocyte interactions in vivo may be achieved by further studies of the mechanism of action of r-IFN-γ on cultured keratinocytes and that the results may provide insight into the pathophysiology leading to a number of common inflammatory and neoplastic skin diseases.

In a number of skin diseases including lichen planus (LP), graft-versus-host disease (GVHD), and mycosis fungoides, keratinocytes express HLA-DR antigen, a class II major histocompatibility antigen not normally expressed by epidermal keratinocytes [1–4]. The role of DR antigen expression in these diseases is not clear, but it is likely that the expression of this antigen on keratinocytes may be related to the pathogenesis of at least some of these disorders.

Recent evidence from our laboratory shows that incubation of keratinocytes with human recombinant gamma interferon (r-IFN-γ) in vitro results in synthesis and expression of HLA-DR by these cells after 3–4 days [5–6]. This induction of DR expression is seen in both transformed and nontransformed keratinocytes [7,8]. Thus, expression of HLA-DR by keratinocytes in situ may be the result of the production of significant quantities of gamma interferon (γ-IFN) by proliferating, activated T lymphocytes in vivo. In vitro, the proliferation of both transformed and nontransformed keratinocytes is inhibited by r-IFN-γ in a concentration dependent fashion [7,8]. This inhibition of growth is maintained for at least 14 days after a short (30 min) pulse of r-IFN-γ in squamous cell carcinoma cell (SCL-1) cultures [8].

In order to better understand the interaction(s) of r-IFN-γ with keratinocytes, we have defined the time course of DR expression on normal cultured keratinocytes after a 4-day pulse of r-IFN-γ. Furthermore, we have determined whether protein(s) other than DR also are induced by r-IFN-γ in these cells.

MATERIALS AND METHODS

Antibodies

A murine monoclonal antibody (L243, an IgG2a) directed against the HLA-DR antigen (Becton Dickinson Monoclonal Center, Inc., Mountain View, California) was diluted 1:10 in 5% heat-inactivated fetal calf serum (FCS) in Dulbecco's phosphate buffered saline, plus 0.02% sodium azide (5% FCS-DPBS). Leu-1 (Becton Dickinson) was used as an isotype control. Fluorescein isothiocyanate conjugated (FITC) rabbit antimouse IgG (R/M-FITC) was obtained from Miles Laboratory, Elkhart, Indiana.

Interferon

Human r-IFN-γ made in *E. coli* was generously supplied by Dr. G. Burton (Genentech, San Francisco, California). The titer of the r-IFN-γ as determined by virus inhibition plaque assay was 5×10^7 reference Units/ml (U/ml) and the specific activity was 10^7 U/mg.

Preparation of Dispersed Skin Cells

Single cell suspensions of normal skin were prepared from facial skin obtained at surgery [9]. Trimmed skin was cut into 1 × 5 cm strips and split-cut with a Castroviejo keratotome set at 0.1 mm. The resulting slices were treated for 25 min at 37°C with 0.3% trypsin plus 0.1% EDTA in GNK (0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.084% $NaHCO_3$, pH 7.3). Dispersed cells were suspended in Dulbecco's Modified Eagles medium supplemented with 10% heat inactivated FCS, 50 μg/ml gentamicin, and 2 mM L-glutamine, termed complete growth medium (CGM). Viability, as determined by trypan blue exclusion immediately after trypsinization, was 90% or better. Two × 10^6 small, round viable keratinocytes were seeded on 3.5 cm collagen-coated Petri dishes (Lux, Flow Lab.) in CGM [9]. The cells were maintained in a humidified incubator with 5% CO_2/95% air at 37°C. For the experiment monitoring DR expression, the r-IFN-γ was added at 48 h after cell seeding and the medium changed 4 days later. The cells were washed 2× with CGM and incubated in 2 ml of CGM without r-IFN-γ. All time points indicated for harvesting of cultures for antibody labeling refers to this time point as day 0.

Antibody Labeling and Fluorescence-Activated Cell Sorter (FACS) Analysis

The cultured cells were washed with Dulbecco's phosphate buffered saline (DPBS) and the monolayer then was trypsinized for 10 min using 0.3% trypsin plus 1% EDTA and resuspended in CGM. The cells were washed once with 5% FCS-DPBS and aliquots of 5 × 10^5–10^6 viable cells were stained for 25 min on ice with anti-HLA-DR monoclonal antibody L243 (1 μg in 50 μl per 10^6 cells). Nonspecific and Fc receptor-mediated background staining was determined either by omitting the first step antibody, anti-HLA-DR, or substituting an isotype control antibody, Leu-1, for the first step. The cells were then washed once with 5% FCS-DPBS and stained for 25 min with 50 μl of a 1:150

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Abbreviations:
CGM: complete growth medium
DPBS: Dulbecco's phosphate buffered saline
FACS: fluorescence-activated cell sorter
FCS: fetal calf serum
5% FCS-DPBS: FCS in DPBS, plus 0.02% sodium azide
FITC: fluorescein isothiocyanate
GVHD: graft-versus-host disease
γ-IFN: gamma interferon
IL-1: interleukin 1
KLR: keratinocyte-lymphocyte reaction
LP: lichen planus
2ME: 2-mercaptoethanol
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
r-IFN-γ: recombinant human gamma interferon
R/M: rabbit antimouse IgG
SDS: sodium dodecyl sulfate
U/ml: Units/ml

dilution of R/M-FITC (Miles Laboratories, Elkhart, Indiana). The labeled cells were washed twice with 5% FCS-DPBS and fixed with 1% paraformaldehyde in phosphate buffered saline (PBS). The fluorescence per cell was determined with a fluorescence-activated cell sorter (FACS III; Becton-Dickinson) and a histogram showing the number of stained cells against the intensity of fluorescence was recorded.

[^{35}S]Methionine Labeling and One-Dimensional Polyacrylamide Gel Electrophoresis (PAGE)

In order to determine whether r-IFN-γ treated keratinocytes synthesize new proteins, we seeded keratinocytes and 3 days later incubated the cultures with and without 100 U/ml r-IFN-γ for 48 h. During the last 18 h, the medium was changed to methionine-free medium containing 0.5% FCS and [^{35}S]methionine was added (10 μCi/ml). The cells were trypsinized and the cell suspensions added to serum to stop trypsin activity. The cells were collected by centrifugation, washed once with PBS and resuspended in 90 μl PBS containing 10 μg/ml DNase I, 10 μg/ml RNase, 1 mM phenyl methane sulphonyl fluoride, 1 mM N-methylmaleimide, 5 mM Mg_2SO_4 and 10 μl 10% triton X-100 in PBS added with mixing. After centrifugation the supernatant was carefully removed as the triton soluble fraction. The pellet was re-extracted successively with 50 mM citrate buffer pH 2.65 (CASC fraction) in the cold and 2% sodium dodecyl sulfate (SDS), 2.5% 2-mercaptoethanol (2-ME) in Tris pH 6.8 (sample buffer) at 100°C for 10 min. Protein factions were brought to 2% SDS, 2.5% 2-ME, by addition, if necessary, of an equal volume of 2× sample buffer and boiled for 10 min. Lane loads were adjusted to the same total activity.

One-dimensional polyacrylamide gel electrophoresis was carried out using the discontinuous system of Laemmli at a constant current of 200 mA/cm^2 until the tracking dye (bromophenol blue) reached the bottom of the gel [10]. Gels were fixed in 50% methanol 10% acetic acid water, soaked in EnHance, washed for 1 h in water, dried, juxtaposed to Kodak X-Omat film and stored in the dark at −70°C for 6–72 h depending on the radioactivity in the gel and then developed.

RESULTS

Time Course of the Maintenance of HLA-DR Expression on Keratinocytes after Removal of r-IFN-γ from the Cultures

We determined whether keratinocytes continue to express HLA-DR in the absence of r-IFN-γ (Table I). Normal, human keratinocytes were incubated for 4 days with or without 300 U/ml r-IFN-γ. At the end of this incubation, the cultures were washed 2× with CGM and representative cultures were harvested (t = 0). The remaining plates were reincubated in CGM without r-IFN-γ for 2, 6, and 8 days. At these times, the cells were trypsinized, stained with anti-HLA-DR antibody plus R/M-FITC and analysed with the FACS. After 2 days incubation without r-IFN-γ approximately the same proportion of keratinocytes continued to express DR antigen as at the time when r-IFN-γ was removed from the cultures (Table I). By day 6, the number of cells expressing DR and the mean fluorescence per cell had decreased markedly. Thus, the proportion of cells expressing HLA-DR declined from 56% at day 0 to 9% at day 8 and the mean fluorescence per cell decreased from 122 units to 15 units over the same time interval. A similar time dependent loss of DR expression after removal of r-IFN-γ was seen in 2 other, separate experiments.

TABLE I. *Duration of HLA-DR expression by human keratinocytes after removal of r-IFN-γ from the culture medium*

Days after removal of r-IFN-γ[a]	Expression of HLA-DR antigen on keratinocytes[b]	
	Proportion of positive cells (% total cells)	Mean fluorescence/cell[c]
0	56	122
2	49	80
6	10	14
8	9	15

[a] Time in culture after removal of r-IFN-γ.

[b] Cells were trypsinized from the culture plate, washed, stained with monoclonal anti-DR antibody plus R/M-FITC and analyzed with the FACS using a linear scale. The percentage of stained cells compared to the total number of cells was determined as was the mean fluorescence per individual cell. The background staining (cells labeled with an isotype control (Leu-1) plus R/M-FITC) was subtracted, as was the value for control cells (not treated with r-IFN-γ at any time), harvested the same day and stained with anti-HLA-DR antibody.

[c] Expressed in arbitrary units.

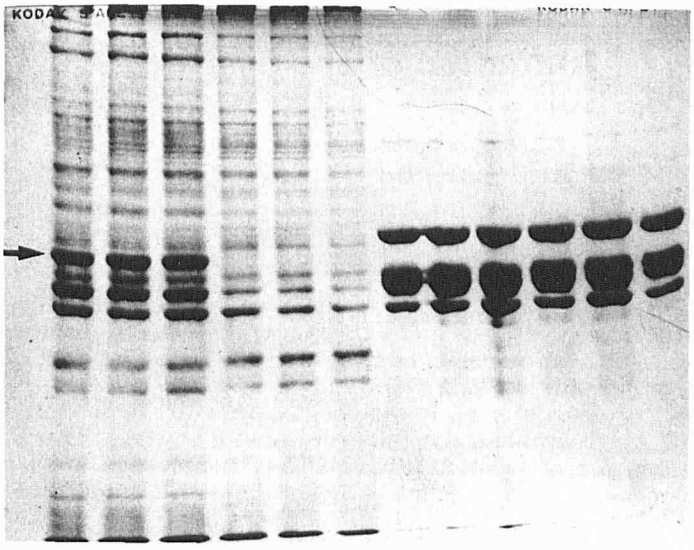

FIG 1. One-dimensional SDS polyacrylamide gel of proteins from [^{35}S]methionine-labeled cultured keratinocytes. The 6 lanes on the *left* contain triton soluble proteins, the 6 lanes on the *right* contain structural proteins. (The *arrow* points to lane #1). Lanes 1–3 and 7–9 contain replicate protein aliquots from r-IFN-γ treated cultures, lanes 4–6 and 10–12 were run with proteins extracted from control cultures. The r-IFN-γ treated cultures demonstrate a heavily labeled band with an apparent molecular weight of 53,000 (→). The control cultures do not show a band in this area. The electrophoretic pattern of the structural proteins (lanes 7–12) shows no differences between r-IFN-γ treated and control cultures.

Gamma Interferon Induces a New Triton Soluble Protein in Keratinocytes

The triton soluble fraction of r-IFN-γ treated cells demonstrated the presence of newly synthesized proteins (other than HLA-DR) not present in untreated cultures (Fig. 1). A major band on SDS-PAGE gels had an apparent molecular weight of about 53,000. Thus, r-IFN-γ induces the synthesis of at least one nonstructural protein, other than HLA-DR antigen. No differences were seen in the molecular pattern of the structural proteins of r-IFN-γ treated and control keratinocyte cultures (Fig 1).

DISCUSSION

Recombinant γ-IFN induces HLA-DR antigen on normal human keratinocytes in vitro and after removal of the lymphokine, the keratinocytes continue to express DR for at least 2 days. By day 6, the proportion of DR-positive cells has decreased to near background levels. Loss of cell surface staining as reflected by mean fluorescence per cell may represent discontinued synthesis or shedding of HLA-DR or internalization/degradation of the antigen. In another system, it has been shown that synthesis of alpha interferon induced HLA proteins decreased markedly 48 h after removing the interferon from the cultures [11]. Further studies aimed at the demonstration of synthesis of mRNA for r-IFN-γ induced proteins in keratinocytes are currently in progress in our laboratory (J. N. Mansbridge, in preparation). Since DR expression persists on the cultured keratinocytes after removal of r-IFN-γ, it may be possible to use these cells as stimulator cells for allogeneic lymphocytes in the keratinocyte-lymphocyte reaction (KLR), an assay for expression of functional HLA-DR antigen on the cell surface. Experiments are in progress to determine the

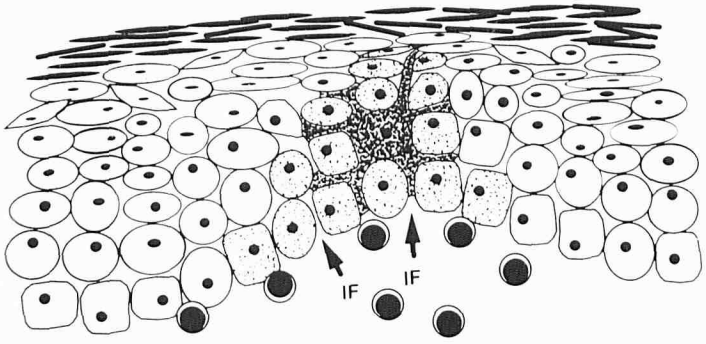

FIG 2. Schematic diagram of the human epidermis showing an "activated" Langerhans cell (*darkly stippled*) extending dendritic processes around neighboring keratinocytes. The keratinocytes and Langerhans cell produce IL-1 which attracts T cells from the peripheral circulation. The T cells (●) in this infiltrate synthesize gamma interferon (*IF*) which leads to synthesis and expression of HLA-DR antigen on the keratinocytes (*lightly stippled cells*) in the immediate vicinity.

response of allogeneic lymphocytes to DR expressing keratinocytes in the KLR (B. J. Nickoloff et al, in preparation).

The r-IFN-γ causes the synthesis of new soluble proteins, other than DR which is identifiable on gels as 2 polypeptides with molecular weights of 28,000 and 34,000 [12]. Using two-dimensional PAGE we are further defining the molecular patterns of the induced proteins (J. N. Mansbridge, in preparation). One of these, the 53,000 dalton protein described in this paper, is a major keratinocyte product representing about 8% of [^{35}S]methionine incorporation into triton-soluble protein or 3% of total protein, at saturating concentrations of γ-interferon (J. N. Mansbridge, unpublished results). Its role in the keratinocyte response to γ-interferon is not known, however it may play a role in the inhibition of keratinocyte growth or may serve as a signal for neighboring cells which, in turn, may lead to skin pathology in vivo. Previous investigations using fibroblasts have also demonstrated the induction of new proteins [13].

One of the skin diseases in which keratinocytes express HLA-DR in situ is LP [1]. This chronic, papulosquamous disease can occur either as a response to drugs, grafting of foreign cells e.g., in GVHD, or without a definable precipitating event [14]. Histologically, LP shows a heavy, dermal infiltrate of activated T cells, thickened granular layer and destruction of basal cells [15,16]. The majority of the T lymphocytes are of the helper subtype, but the infiltrate also includes a small proportion of cytotoxic/suppressor cells. Langerhans cells are increased in the epidermis [16]. We propose that release of large quantities of γ-IFN by activated dermal T cells is the critical pathologic event which leads to keratinocyte expression of HLA-DR in this disease (Fig 2). In this view, Langerhans cells and/or keratinocytes meet foreign antigen (e.g., virus, drug, allogeneic cells) and produce interleukin 1 (IL-1) [17,18]. This lymphokine attracts circulating T cells to the area and stimulates them to proliferate [19]. T cells in the dermis in turn produce γ-IFN which diffuses into the epidermis causing keratinocyte expression of HLA-DR and decreased proliferation [20]. Moreover, we propose that DR in combination with a foreign antigen expressed on the surface of these keratinocytes is recognized as "nonautologous" by the cytotoxic T cells present at the dermal-epidermal junction [16]. Some of these cells may then attack the keratinocytes resulting in the appearance of the Civatte bodies characteristic of LP [15]. Destruction of the basal cell layer results in a wound healing response [21] which in turn causes the appearance of increased numbers of Langerhans cells (M. Reusch, in preparation) and more IL-1 is produced. In this way, the process becomes self-perpetuating.

If this view of the etiology of LP is correct, we would predict that inhibitors of γ-IFN production or blocking of γ-IFN action on the target cells (i.e., keratinocytes) would be therapeutically efficacious in LP. Similarly, a local inhibitor of IL-1 production or a blocker of IL-1 receptors on T cells should also interrupt the pathologic events leading to clinical expression of LP.

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