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Class II MHC antigen expression by human keratinocytes results from lympho-epidermal interactions and γ-interferon production

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SUMMARY

Human peripheral blood lymphocytes were co-cultured with either allogeneic, autologous or purified protein derivative of tuberculin (PPD) pulsed autologous epidermal cells. In these mixed skin cell-lymphocyte culture reactions (MSLR), lymphocytes are stimulated to proliferate by epidermal cells. The supernatants of MSLR were examined for their capacity to induce class II MHC antigen expression on separately cultured epidermal cells. It is shown that supernatants from allogeneic and PPD pulsed autologous MSLR contained the factor(s) which stimulated HLA-DR antigen synthesis and expression by 30–40% of cultured epidermal cells. Kinetic analysis revealed a production rate maximum between 72 and 96 h of lympho-epidermal co-cultures. The factor mediating the induction of HLA-DR antigen expression on epidermal cells is thought to be γ-interferon, because it was sensitive to pH 2 as well as heat incubation. Furthermore, anti-γ-interferon monoclonal antibody abolished its activity. It is proposed, that HLA-DR antigen expression by keratinocytes observed *in vivo* in different dermatological inflammatory disorders originates from lympho-epidermal interactions and local γ-interferon production as documented here in experiments *in vitro*.

Keywords Class II MHC antigens keratinocytes MSLR

INTRODUCTION

Class II major histocompatibility complex (MHC) encoded molecules are cell surface glycoproteins that are involved in various phases of an immune response. Although their expression is generally considered to be restricted to the cells of the immune system, it is now known that a variety of endothelial and epithelial cell types can be induced to express HLA-DR (Ia-like) antigens (Natali *et al.*, 1981; Basham & Merigan, 1983; Pober *et al.*, 1983).

In normal human epidermis, HLA-DR antigens are expressed only by dendritic Langerhans cells (LC) which represent no more than 2–3% of all epidermal cells (Rowden, Lewis & Sullivan, 1977; Stingl *et al.*, 1978). Recent studies, however, have shown that under certain experimental conditions and particularly in some dermatological disorders, the keratinocytes, a major component of the epidermis, can be induced to express HLA-DR antigens. These skin diseases include allergic contact dermatitis (Barclay & Mason, 1982), mycosis fungoides (Tjerlund, 1978), lichen planus (Tjerlund, 1980), graft versus host disease (Lampert, Suitters & Chisholm, 1981) and the tuberculin skin reaction (Scheynius & Tjerlund, 1984). The reports presenting class II MHC antigen expression by keratinocytes in other dermatoses represent a rapidly expanding field

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(Lampert, 1984). Recently, it has also been demonstrated that HLA-DR antigen is not transferred by neighboring LC but that the keratinocytes are synthesizing it themselves (Volc-Platzer *et al.*, 1984). One of the common features of diseases accompanied by keratinocyte HLA-DR expression is the presence of a relatively dense, predominantly lymphocyte infiltrate. This lymphocytic infiltration is located in the dermis as well as in the epidermis and is composed of various lymphocyte subpopulations.

The close contact between lymphocytes and epidermal cell components observed in these pathological states, leads to the creation of a model to study *in vitro* the lympho-epidermal interactions (Main *et al.*, 1971; Stingl *et al.*, 1978; Braathen *et al.*, 1980).

In this approach, peripheral blood lymphocytes may be co-cultured with either allogeneic, autologous or antigen pulsed autologous epidermal cells. This mixed skin cell-lymphocyte culture reaction (MSLR) has already been used, in both laboratory animals and humans, to demonstrate the LC antigen presenting capacities as well as the reactive T lymphocyte subsets (Stingl *et al.*, 1978; Braathen *et al.*, 1980; Czernielewski *et al.*, 1982).

The purpose of the present study was to determine whether during lympho-epidermal interactions *in vitro* a factor or factors inducing HLA-DR expression on keratinocytes may be generated, as suggested by observations *in vivo*.

MATERIAL AND METHODS

Cells. Healthy young adult volunteers provided skin and venous blood samples. Peripheral blood mononuclear cells were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Böyum, 1976). The cells were washed three times with 0·9% NaCl and resuspended in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) containing 20% heat inactivated AB human serum, 2 mM L-glutamine and antibiotics (complete medium). Adherent cells were removed by incubation on culture plastic Petri dishes (90 min, 37°C, 5% CO_2). Harvested non-adherent cells contained fewer than 1% esterase positive or latex-ingesting monocytes. After harvesting, the cells were washed and resuspended to the desired concentration in complete medium.

Human epidermal cells were isolated by trypsin digestion of keratotomized skin slices (0·25% trypsin, 37°C, 45 min) as previously described (Liu, Eaton & Karasek, 1979). The resultant dispersed cells were filtered through a sterile gauze and resuspended in complete medium. They did not contain dermal endothelial cells or fibroblasts as evaluated by anti-factor VIII antibody staining and morphological criteria, respectively. Viability, as determined by trypan blue exclusion, was greater than 90% immediately after trypsinization.

Mixed skin cell-lymphocyte cultures. These were conducted as previously described (Czernielewski *et al.*, 1982; Bagot *et al.*, 1985). Briefly, 10^5 lymphocytes were co-cultured with 10^5 irradiated epidermal cells (2600 rads) in round-bottomed microtitre plates (Linbro Chemical Co. New Haven, CT) for 6 days. All cultures were carried out in triplicate. ^{3}H-Thymidine, 1 μCi/well, was added 18 h before cell harvesting. Cells were collected with a Mash II automated cell harvester and the amount of incorporated thymidine was determined by liquid scintillation counting. Results are expressed as mean counts per minute ct/min $\pm$ s.d. As controls, lymphocytes and epidermal cells were cultured alone.

Either autologous or allogeneic co-cultures were performed. For PPD specific proliferations, tuberculin-purified protein derivative (Institut Pasteur, Paris, France) was added (0·01 mg/ml) at the beginning of the co-cultures.

Preparation of MSLR supernatants. In parallel, the bulk lympho-epidermal co-cultures were performed: 10^6 epidermal cells and 10^6 lymphocytes were co-cultured in different combinations in 2 ml of complete medium in 24-well Costar plates (Costar, Cambridge, MA) under culture conditions otherwise identical to the microplate cultures. At the end of the culture period, the supernatants were harvested, filtered through 0·2 μm pore size filters (Milipore, Bedford, MA), frozen and stored at -80°C. In additional experiments, supernatants were harvested from cultures every 24 h.

Testing of supernatants for HLA-DR inducing capacities. Primary cultured, human adult

epidermal cells grown to confluency on 3·5 cm collagen-coated Petri dishes were used. Different MSLR final supernatant concentrations as well as supernatants harvested every 24 h from MSLR or controls were added to the confluent epidermal cell cultures. After 96 h incubation, the epidermal cells were harvested by means of 0·3% trypsin plus 1% EDTA and tested for HLA-DR expression. They were stained with anti-HLA-DR monoclonal antibody (Becton Dickinson, Mountain View, CA) followed by fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Tago, Burlingame, CA). After subsequent washes, the cells were processed for flow-cytometry measurements (FACS IV) and the number of cells expressing HLA-DR specificity was determined.

Untreated epidermal cells or epidermal cells treated with supernatants from separately cultured lymphocytes were the controls.

Blocking of supernatant activity. The MSLR supernatants were dialysed for 24 h against 0·1 M glycine-HCl, 0·15 M NaCl, pH 2·0, buffer followed by dialysis for 48 h against phosphate-buffered saline, pH 7·2, and a final dialysis against complete medium. As controls, supernatants were dialysed in phosphate-buffered saline and complete medium.

Supernatant samples were also analysed for heat stability by incubating them at 56°C for 2 h, followed by cooling on ice and testing for HLA-DR antigen inductive capacities.

Monoclonal antibody to human γ-IFN was provided by Chemicon International Inc. Los Angeles, CA, with a neutralizing titre of 2×10^4 U/ml. Sheep antiserum to human α-IFN (Interferon Science Inc. New Brunswick, NJ) had a neutralizing titre of 10^5 U/ml.

Both antibodies were incubated with MSLR supernatants for 2 h at 37°C before their addition to epidermal cell cultures. Five thousand neutralizing U/ml for anti-α-IFN and 500 neutralizing U/ml for anti-γ-IFN antibody were used. As controls, supernatants were treated with swine anti-human immunoglobulins antiserum (Nordic, Tilburg, Netherlands).

RESULTS

Human epidermal cells stimulate the proliferation of peripheral blood lymphocytes in MSLR. Results from typical experiments comparing allogeneic, autologous and PPD pulsed autologous MSLR are depicted in Table 1. As expected from a previous work (Czernielewski *et al.*, 1982; Braathen & Thorsby, 1982), significant stimulation of lymphocytes was observed with either allogeneic or PPD pulsed epidermal cells. In autologous combinations without PPD, lymphocyte responses were less effective: however, they were always greater than that of separately cultured lymphocytes.

Table 1. Lymphocyte responses in either allogeneic, autologous or PPD pulsed autologous mixed skin cell-lymphocyte culture reactions (MSLR)

	Proliferative responses (mean ct/min ± s.d.)	
MSLR types and controls	Experiment No. 1	Experiment No. 2
Allogeneic MSLR	74,037 ± 3,569	66,933 ± 6,189
L* alone	650 ± 131	1,234 ± 405
EC† alone	1,231 ± 321	691 ± 148
Autologous MSLR	4,281 ± 832	5,863 ± 624
L alone	809 ± 321	951 ± 321
EC alone	1,001 ± 395	821 ± 408
Autologous MSLR + PPD	118,414 ± 4,833	145,100 + 6,208
L alone	480 ± 247	480 ± 247
EC alone	3,509 ± 264	677 ± 87

* L, peripheral blood lymphocytes.
† EC, epidermal cells.

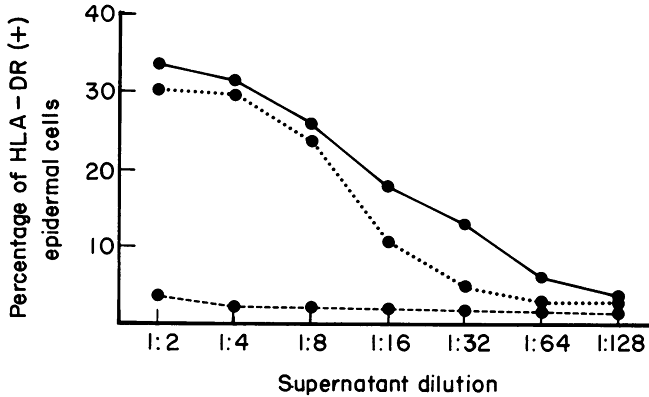

Figure 1. Percentage of HLA-DR positive epidermal cells after 96 h incubation with different dilutions of supernatants provided from allogeneic (——), autologous (- - - -) or PPD pulsed autologous (· · · ·) MSLR. Control supernatants (from lymphocytes or epidermal cells cultured alone) presented HLA-DR inducing capacity comparable to the supernatants from autologous MSLR. The means of triplicate experiments are given. The standard deviations did not exceed 6%.

Supernatants from allogeneic and PPD pulsed autologous MSLR induce HLA-DR expression on keratinocytes. It seemed pertinent to investigate whether MSLR supernatants were able to induce HLA-DR expression. For this purpose, supernatants from the various MSLR were used to treat primary cultured epidermal cells. After 96 h of treatment, epidermal cells were harvested, stained with anti-HLA-DR monoclonal antibody and the number of positives was determined by FACS analysis. As shown in Fig. 1, supernatants from allogeneic as well as from PPD pulsed autologous MSLR induced HLA-DR antigen expression on epidermal cells in a dose dependent manner. Supernatants from autologous MSLR as well as from controls (lymphocytes or epidermal cells cultured alone) did not demonstrate this activity (1·3–5·0% of HLA-DR(+) epidermal cells).

Untreated cultured epidermal cells demonstrated 0·5–1·5% of HLA-DR positives. It was interesting to investigate at what time during the MSLR, the HLA-DR antigen inductive factor(s) is produced. Consequently, the supernatants harvested at different times from allogeneic MSLR were diluted 1:2 and incubated with cultured epidermal cells. As indicated on Fig. 2, the HLA-DR inductive abilities of supernatants appeared between 72 and 96 h. The supernatants harvested from

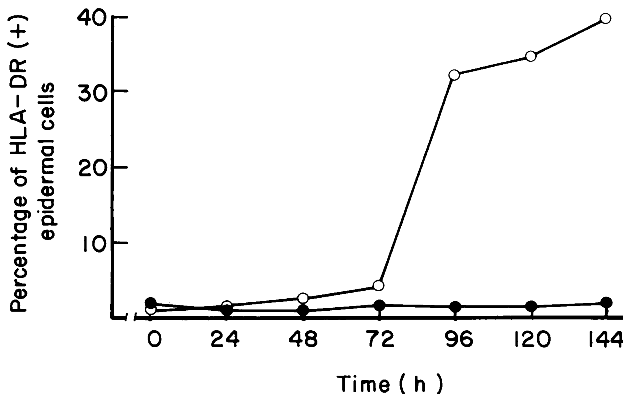

Fig. 2. Kinetics of induction of HLA-DR epidermal cell antigen expression by supernatants harvested at different times from allogeneic MSLR. These supernatants were diluted 1:2 and used for 96 h treatment of primary cultured epidermal cells. Then, epidermal cells were harvested, processed for immunofluorescence and the number of HLA-DR positive cells was estimated by flow-cytometry. The means of triplicate samples are shown. Allogeneic MSLR (○); lymphocytes cultured alone (●).

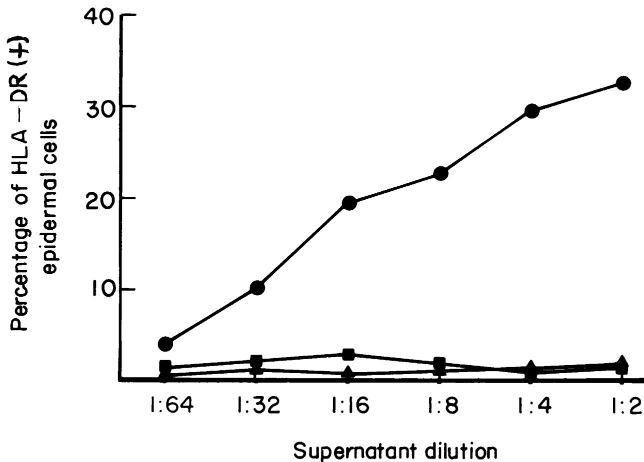

Fig. 3. Effects of pH 2 and 56°C treatments on the HLA-DR antigen inductive abilities of allogeneic MSLR supernatants. These supernatants were treated with pH 2 or heat (see Materials and Methods) and their capacity to induce HLA-DR expression was examined. Non-treated supernatants (●); effects of pH 2 treatment (■); effects of 56°C treatment (▲). Numbers are the means of three determinations.

MSLR at 120 and 144 h demonstrated hardly more (6%) HLA-DR inductive activities compared with those harvested at 96 h.

In supplementary experiments, the addition of cyclohexamide (which blocks protein synthesis) to the epidermal cell cultures treated by MSLR supernatants resulted in the inhibition of HLA-DR expression by epidermal cells. This inhibition was related to the concentrations of cyclohexamide (10^{-6}–10^{-9} g/ml) present in the cultures.

Anti-γ-IFN antibody, pH 2 and 56°C treatments all blocked the activity of MSLR supernatants. Figure 3 shows that pretreatment of allogeneic MSLR supernatants with either pH 2 or 56°C totally abolished the HLA-DR antigen inductive activity. Similar results were obtained with PPD pulsed autologous MSLR supernatants. The supernatants dialysed in phosphate-buffered saline and complete medium retained their activity as did untreated supernatants. Furthermore, preincubation of allogeneic or PPD pulsed autologous MSLR supernatants with anti-γ-IFN monoclonal

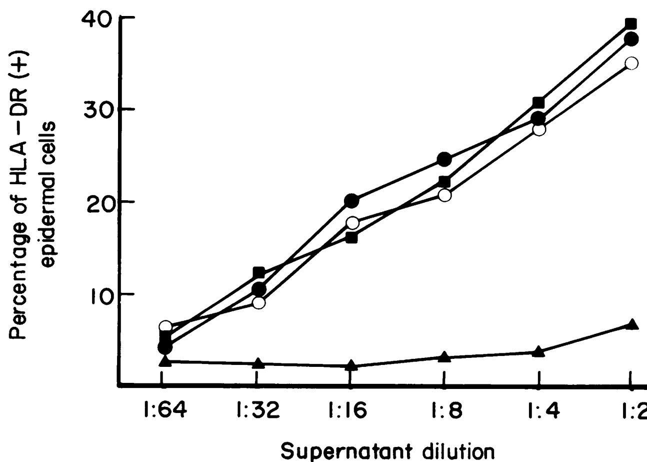

Fig. 4. Effects of antisera against α-, γ-interferon and anti-human immunoglobulins (Ig) on induction of HLA-DR epidermal cell expression produced by PPD pulsed autologous MSLR supernatants. Different supernatant dilutions were preincubated (2 h at 37°C) with either anti-α-IFN, anti-γ-IFN or anti-human Ig antibodies before adding to the epidermal cell cultures. The means of triplicate samples are shown.

antibody (500 neutralizing U/ml) abolished supernatant activity. In contrast, pretreatment of the supernatants under the same conditions with either anti-α-IFN or anti-human immunoglobulins (Ig) antibodies did not have any effect (Fig. 4).

DISCUSSION

A wide variety of dermatological diseases are characterized by infiltration of lymphocytes into the dermis as well as by their invasion into the overlying epidermis. An approach *in vitro* to lympho-epidermal interactions has been provided by co-culture *in vitro* of dissociated epidermal cells with peripheral blood lymphocytes. In this skin cell-lymphocyte culture reaction (MSLR) epidermal cells are recognized by lymphocytes which are stimulated to proliferate. MSLR exhibits characteristics of an immune response such as memory and specificity and is similar to the classical one-way mixed lymphocyte reaction (Tanaka & Sakai, 1979). It is well established that lymphocytes may proliferate upon stimulation with either allogeneic, autologous or antigen pulsed autologous epidermal cells (Czernielewski *et al.*, 1982; Braathen & Thorsby, 1982; Sontheimer, 1983). Furthermore, it is known that of the various types of epidermal cells, Langerhans cells, which bear class II MHC antigens, are crucial for MSLR to occur (Stingl *et al.*, 1978; Czernielewski *et al.*, 1982; Braathen & Thorsby, 1982).

In the present study we confirm the ability of epidermal cells to induce lymphoproliferative responses in various lympho-epidermal co-culture combinations. Furthermore, we studied whether one or several factors able to induce HLA-DR expression on keratinocytes could be generated during lympho-epidermal culture interactions. Only few epidermal cells, namely Langerhans cells, express HLA-DR antigen in non-treated human epidermal cell cultures (Czernielewski, 1985). However, when cultures were incubated with supernatants harvested from either allogeneic or PPD pulsed autologous MSLR, the percentage of positives increased to 30–40%. Supernatants from autologous MSLR did not demonstrate such activity.

The most potent HLA-DR inductive abilities of supernatants were observed between 72 and 96 h of either allogeneic or PPD pulsed autologous MSLR. The supernatants from these two types of MSLR demonstrated also, in classical interferon assay, the ability to inhibit a cytopathogenic effect of vesicular stomatitis virus on human amnion WISH cells (data not shown). Thus, the factor produced during lympho-epidermal interactions *in vitro* was probably γ-IFN according to classical criteria. It was susceptible to low pH (pH 2) and high temperature (56°C) and was not inhibited by antibody to α-IFN. In contrast, anti-γ-IFN monoclonal antibody abolished supernatant activity.

Furthermore, it is in accordance with previous studies demonstrating that the addition of either natural or recombinant γ-IFN to the epidermal cell cultures results in HLA-DR induction on keratinocytes (Czernielewski, 1985; Basham *et al.*, 1984; Volc-Platzer *et al.*, 1985).

It has been demonstrated that T lymphocytes are responder cells in MSLR (Czernielewski *et al.*, 1982). Furthermore, lymphocytes depleted of their $OKT4^+$ subsets failed to respond in MSLR, suggesting that helper/inducer T cells play a major role in MSLR. Consequently, it may be supposed that $OKT4^+$ cells are the main source of the increased production of γ-IFN in MSLR, as is the case in OKT3 monoclonal antibody stimulated lymphocytes (Chang *et al.*, 1982).

The present study demonstrates that γ-IFN is produced during lympho-epidermal interactions *in vitro* and induces class II MHC antigens on primary cultured human epidermal cells. It can, thus, be suggested that a similar mechanism functions *in vivo* in different dermatoses accompanied by both lymphocyte infiltration and HLA-DR expression by keratinocytes.

However, the potential immunological role of keratinocytes or other non-lymphoid cells that express class II MHC antigens is still obscure. It seems that in the case of human keratinocytes, the induction of HLA-DR antigen is not an essential primary event in triggering an immune response (Czernielewski, 1985) but is rather secondary to increased levels of γ-IFN found as part of a generalized release of T lymphocyte-derived lymphokines during lympho-epidermal interactions.

Nevertheless, the possibility that secondary increases in class II MHC antigen expression could aggravate the autodestructive process (i.e. $HLA\text{-}DR^+$ keratinocytes may well be the target for the activated cytotoxic T lymphocytes) cannot be ignored and warrants further experimental consideration.

Recently, it was also suggested by Roberts *et al.* (1985) that Ia^+ keratinocytes are involved in facilitating the movement of lymphoid cells into the skin in response to antigenic stimulation and subsequently could enhance the intensity and duration of contact hypersensitivity responses in mice. Finally, it is also possible that HLA-DR antigen synthesis and expression by keratinocytes, or γ-IFN itself, may influence the production of epidermal cell-derived thymocyte-activating factor (ETAF) and modulate additionally the immunological response in the skin (Luger *et al.*, 1981; Sauder *et al.*, 1984; Sauder, Monick & Hunninghake, 1985).

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