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STUDIES ON HUMAN EPIDERMAL LANGERHANS' CELLS: II. ACTIVATION OF HUMAN T LYMPHOCYTES TO HERPES SIMPLEX VIRUS

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Abstract. Epidermis from patients suffering from recurrent herpes labialis was separated from dermis by means of a suction blister device and dissociated with trypsin. The epidermal cell suspensions obtained were 80–95% viable and contained 3–5% Langerhans' cells, as judged by immunofluorescence staining of the cells with a rabbit anti-DR antiserum. T lymphocytes from the same patients were co-cultured with herpes simplex virus antigen (HSV-Ag) or live virus (HSV), with or without epidermal cells or macrophages. A strong proliferative T cell response to HSV-Ag and HSV was obtained, provided that the cultures also contained epidermal cells or macrophages. Pretreatment of the epidermal cells with a rabbit anti-DR antiserum plus complement abolished the responses, while pretreatment with normal rabbit serum plus complement did not. These data therefore indicate that HLA–DR positive Langerhans' cells are able to present herpes simplex virus in an immunogenic way to T lymphocytes.

Key words: Langerhans' cells; Herpes simplex virus; T lymphocytes; Herpes infection

The epidermal Langerhans' cells constitute a minor cell population among the human epidermal cells. Recent data indicate that they play an important role in the human immune system. They have Fc and C3 receptors (23). They can selectively take up various contact allergens (19) and it was suggested that they form a reticulo-epithelial trap for external contact allergens (17). Langerhans' cells in close apposition to mononuclear cells were frequently seen at sites of positive allergic contact dermatitis, but not in contact primary irritant reactions (19, 20, 21).

The Langerhans' cells also express the HLA–D/DR cell-membrane molecules (11, 15, 24) similar to blood monocytes–macrophages. The HLA molecules can be divided into two classes: the HLA–ABC molecules which are present on all nucleated cells, and the HLA&D/DR molecules which have a more restricted tissue distribution, being present mainly on B lymphocytes, monocytes–macrophages and endothelial cells (30). Both HLA–ABC and D/DR molecules show extensive polymorphism and are strongly antigenic in allogeneic combinations, inducing the allograft response. It has been shown that the D/DR antigens are mainly responsible for the proliferative response of T cells when confronted with allogeneic cells *in vitro*, i.e. the mixed lymphocyte culture (MLC) interaction (28).

In a recent report we demonstrated the ability of HLA–DR positive Langerhans' cells to stimulate allogeneic T lymphocytes (5). Macrophage D/DR apparently also participate in the activation of T cells with foreign antigens such as PPD (1, 2, 27), herpes simplex virus (4) and haptens such as TNP (29), i.e. these responses are restricted by the self-HLA—D/DR determinants of the T cell donor. Similarly, we were recently able to demonstrate that epidermal Langerhans' cells can substitute for macrophages in inducing a PPD-specific response of T cells from sensitized donors (5).

Here we report that human epidermal cells which express the D/DR antigens can also substitute for macrophages in inducing a herpes simplex virus specific response of T cells from sensitized donors. The responsible cells are in all probability the Langerhans' cells.

MATERIALS AND METHODS

Patient material

Epidermal cells and blood samples were obtained from patients with recurrent herpes labialis. The state of sensitization was confirmed by the presence of complement-binding antibodies with a titre >8 or neutralizing antibody titre >4. Complement binding reaction was performed by the standard technique with commercially available antigen (Orion Diagnostica, Helsinki, Finland). The neutralizing test was performed using 100 tissue culture infectious

Table 1. *Epidermal cell (Ec) and macrophage (Mϕ) dependent T lymphocyte response to herpes simplex antigen (HSV-Ag) and herpes simplex live virus (HSV) in vitro*

Exp. no.	T[a] cells		Ec[b]		T+Ec		Mϕ[c]		T + Mϕ	
HSV-Ag	−	+	−	+	−	+	−	+	−	+
1	97±33[d]	171±76	65±28	50±23	231±38	2 941±650	64±12	42±7	179±58	3 393±100
2	124±26	293±154	52±27	80±39	675±105	14 407±780	134±56	117±17	66±29	5 236±113
3	116±43	527±23	56±19	141±16	533±112	13 336±831	148±31	79±15	351±136	6 343±1 368
HSV	−	+	−	+	−	+	−	+	−	+
2	124±26	166±84	52±27	49±11	675±105	3 238±152	134±56	165±27	66±29	776±353
3	116±43	400±94	56±19	30±10	533±112	9 684±1 410	148±31	28±13	351±136	4 848±209
4	150±54	715±100	45±18	119±36	137±12	3 914±423	−	−	−	−

[a] 5×10^4 T cells.
[b] 5×10^4 epidermal cells.
[c] 5×10^3 macrophages.
[d] Mean cpm ± S.E.

doses of HSV hominis type 1, grown on Vero cells. All serology testing was performed by Dr M. Degré, Institute of Bacteriology, The National Hospital, Oslo.

Rabbit anti-DR antiserum and normal rabbit serum

The anti-DR antiserum preparation is described elsewhere (22). It was absorbed with pooled human erythrocytes before use.

Herpes simplex virus antigen (HSV-Ag)

We used lyophilized herpes simplex type 1 CF (complement fixation) antigen produced in monkey kidney cells (Orion Diagnostica, Helsinki, Batch DI, Cat. no. D428). The control antigen used (Orion Diagnostica, Batch DI, Cat. no. D711) was processed corresponding to the CF antigen, but it did not contain herpes simplex type 1 virus antigen.

Herpes simplex virus type 1 (HSV)

Live herpes simplex virus type 1 was kindly provided by Dr M. Degré, Institute of Bacteriology, The National Hospital, Oslo. The herpes simplex virus used was grown on Vero cells. It contained 10^6 tissue culture infectious doses per ml.

Peroxidase staining

Peroxidase staining of epidermal cell suspensions was performed with cytocentrifuge (Shandon Scientific Co., Ltd., London) preparations as described by Kaplow (9).

Latex ingestion

One hundred μl latex (Bacto Latex 0.81, Difco Laboratory, Michigan, USA) was added to 10 ml RPMI 1640. Cell suspensions were adjusted to 1×10^6 per ml. Equal parts of cell and latex suspensions were incubated for 30 min at 37°C and the number of latex-ingesting cells per 100 was counted.

Epidermal cells (Ec)

Epidermis was separated from dermis at the basement membrane *in vivo* by means of a suction blister device (10). The sheets of epidermis were then dissociated by means of trypsin, essentially as described by Stingl et al. (23). After washings, the cells were resuspended in medium RPMI 1640 with L-Glutamine (Bio-Cult, Gibco, Glasgow, Scotland) supplemented with penicillin, streptomycin and and 20% normal human serum. Viability was checked by trypan blue exclusion and ranged from 80 to 95%, but was usually more than 90%. The cell suspensions contained 3–5% Langerhans' cells, as judged by immunofluorescence staining of the cell suspensions with a rabbit anti-DR antiserum. The staining was performed by first incubating the cell suspensions with the anti-DR antiserum at 4°C for 30 min and then, after two washings, the cells were incubated another 30 min at 4°C with an FITC-conjugated swine anti-rabbit antiserum (DAKO-immunoglobulin, Copenhagen, Denmark). After final washings, the cells were counted in an immunofluorescence microcope.

Blood cell separation techniques

Defibrinated blood was separated by the Ficoll-Isopaque flotation technique (6) using Lymphoprep (Nyco, Oslo, Norway).

T cell preparation

The separated cells were suspended in tissue culture medium RPMI 1640 with 100 I.U. penicillin/ml, 100 μg/ml streptomycin and 20% normal serum from a pool of healthy blood donors, and incubated in 25 cm^2 flat-bottomed tissue culture flasks (Cat. no. 3013, Falcon, Calif., USA) for 2 h at 37°C. Following this incubation, the non-adherent cells were pipetted off and made to form rosettes with AET-treated sheep red blood cells (SRBC) (12). The adherent cells were incubated further (see later). The rosettes were resuspended in RPMI 1640 without serum and separated from "non-T cells" by Ficoll-Isopaque flotation. The SRBC were lysed with normal serum containing anti-sheep zeno-antibodies, washed twice and resuspended in tissue culture medium. This cell suspension was incubated in flat-bottomed tissue culture flasks for 90 min in order to remove further remaining adherent cells. Thereafter the non-adherent cells were pipetted off and washed twice in RPMI 1640. The adherent cells from this preparation were discarded.

In order to obtain pure T cell populations, the non-adherent cells were again made to form rosettes with AET-

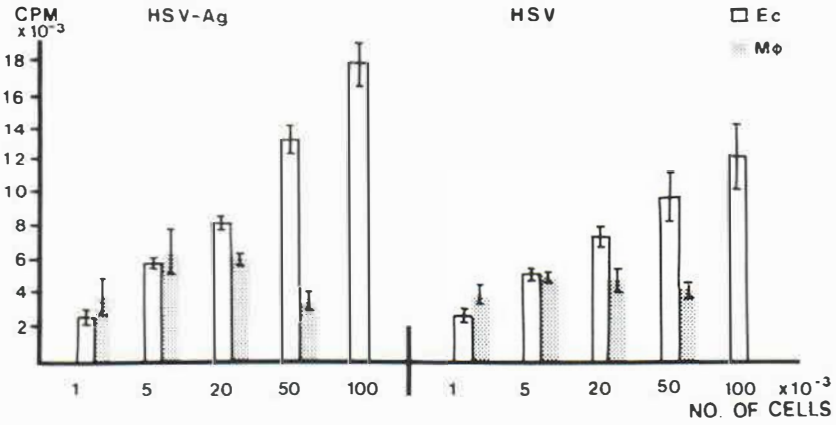

Fig. 1. Influence of the number of epidermal cells (*Ec*) and macrophages (*Mø*) added to the cultures on the T cell response to herpes simplex type I virus antigen (*HSV-Ag*) and to live herpes simplex virus type I (*HSV*). Values are in mean cpm ± S.E.

treated SRBC. After lysing the SRBC with normal serum, the cells were washed twice, resuspended in tissue culture medium and adjusted to 1×10^6 cells/ml. This cell suspension was designated "T cells". More than 80% of these formed rosettes with AET-treated SRBC, less than 2% ingested latex and less than 1% were Ig-positive. Usually 95% or more of the cells proved viable by means of trypan blue exclusion.

Preparation of adherent cells/macrophages

The adherent cells were released by incubation with Medium RPMI 1640 supplemented with 30% normal human serum and 3.33 mg per ml EDTA for 90 min on ice before being removed by forcible pipetting. After two washings at 4°C, the cells were kept on ice until used. Of these adherent cells, designated "Macrophages" (Mφ), more than 85% ingested latex and the viability was usually more than 95% as determined by trypan blue exclusion. To inhibit any contributing proliferation from the adherent cells, these were always irradiated with 2 000 rad before use, using a ^{137}Cs unit.

Cell culture techniques

Cell cultures were incubated in round-bottomed microtitre plates (Model 15-MRC-96, Linbro Scientific, Inc., Hamden, Conn., USA) in a humid 5% CO_2 atmosphere at 37°C for 5 days. 5×10^4 T lymphocytes were co-cultured with or without various numbers of adherent cells or epidermal cells and with and without HSV-Ag or HSV. Each well contained 170 µl Medium RPMI 1640 supplemented with antibiotics and 20% normal human serum. ^{3}H-[thymidine] was added 18 h prior to harvesting, which was performed with a semi-automatic multiple cell culture harvester (Skatron, Lierbyen, Norway). Incorporation was measured by means of a liquid scintillation counter and expressed as mean cpm ± S.E. of triplicates.

Treatment of epidermal cell suspensions with a rabbit anti-DR antiserum or normal rabbit serum plus complement

This was performed in two ways. In some experiments, 5×10^6 epidermal cells were incubated in 0.3 ml of a rabbit anti-DR antiserum or normal rabbit serum (previously absorbed with pooled human erythrocytes) diluted 1 : 10 at 37°C for 30 min before 0.3 ml of normal rabbit complement, diluted 1 : 5, was added and the incubation prolonged for a further hour at 37°C. The cells were then washed three times and resuspended in Medium RPMI 1640 supplemented with penicillin, streptomycin and 20% normal human serum. Cell count and viability were checked and adjusted to total viable cells before they were used in experiments.

In other experiments, the rabbit anti-DR or the normal rabbit serum and complement were added in a final dilution of 1 : 20 to the wells containing epidermal cells, and allowed to react for 1 h at 20°C before the purified T cells and HSV-Ag or HSV were added.

RESULTS

Peroxidase staining of epidermal cell suspensions

Since our results depend heavily upon an eventual monocyte–macrophage contamination of the epidermal cell suspensions used, we investigated whether monocytes were present. Several cytocentrifuge preparations were made of each of three different epidermal cell suspensions and stained with peroxidase to detect monocytes. More than 1 000 cells from each suspension were examined, but no stained cells were detected.

Epidermal cell and macrophage-dependent autologous T lymphocyte response to HSV-Ag and HSV

Epidermal cells or macrophages were co-cultured with autologous T lymphocytes and HSV-Ag or HSV as described. Eight experiments were performed. Table I shows the results of typical experiments using four different cell donors and HSV-Ag or HSV. In the cultures containing T cells

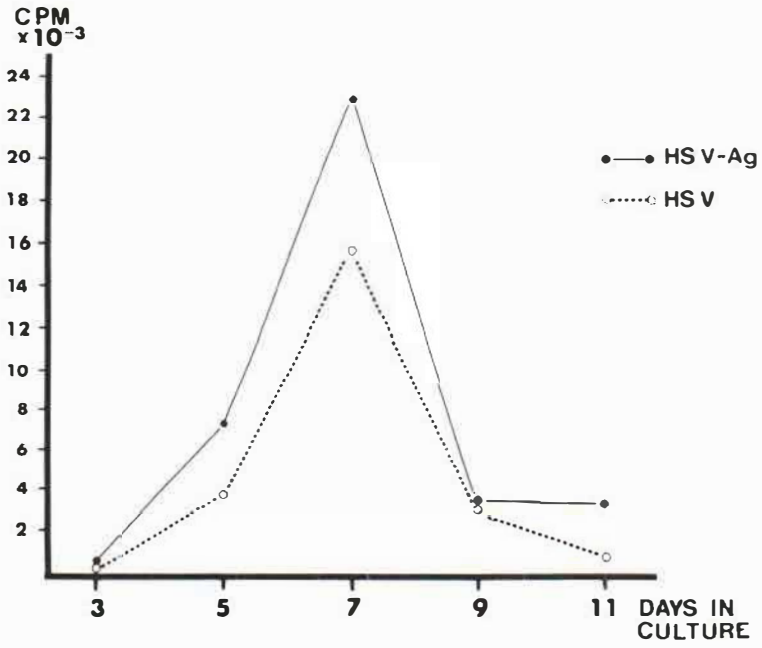

Fig. 2. Kinetics study of the epidermal cell-dependent autologous T lymphocyte response to herpes simplex virus type I antigen (HSV-Ag) and to live herpes simplex virus type I (HSV), expressed as median cpm.

and epidermal cells or macrophages with HSV-Ag or HSV, a strong proliferative T cell response was obtained. T cells, epidermal cells, or macrophages co-cultured with HSV-AG or HSV alone did not respond, indicating that the presence of epidermal cells or macrophages in the cultures was necessary to induce the T cell response to HSV-Ag and HSV.

Studies using epidermal cells pulsed with HSV-Ag or HSV

In some experiments epidermal cells were incubated with HSV-Ag or HSV for 90 min at 37°C and washed three times before being added to the cultures. No qualitative differences in the responses were found between these experiments (data not shown) and those with HSV-Ag or HSV added to the wells. This indicates that antigen was bound to the epidermal cells within 90 min.

Influence of number of epidermal cells or macrophages

In some experiments, various numbers of epidermal cells or macrophages were added to 5×10^4 T cells, increasing from 1×10^3 cells to 1×10^5 per well. Fig. 1 shows a typical experiment. Both the HSV-Ag and the HSV T cell response were maximal with 10% macrophages added, while the response in cultures containing epidermal cells increased with the number of epidermal cells added, up to the highest tested number of 1×10^5 epidermal cells, i.e. an Ec-T cell ratio of 2:1. The macrophages induced a higher response than the epidermal cells up to 5×10^3 cells added, but thereafter the epidermal cells induced the highest response, and the maximum response obtained was substantially higher with epidermal cells than with macrophages.

Kinetics of epidermal cell-dependent T cell response to HSV-Ag and HSV

These experiments were performed with HSV-Ag- and HSV-pulsed epidermal cells as well as with HSV-Ag and HSV directly added to the cultures, using the same T cell donors. Both approaches gave essentially the same results. In Fig. 2, the results with HSV-Ag or HSV added to the cultures are presented. A peak response was obtained on day 7, both for HSV-Ag and HSV.

Epidermal cell-dependent T cell response to HSV-Ag and HSV is abolished by pretreatment of epidermal cells with a rabbit anti-DR antiserum plus complement

In some experiments, epidermal cells were pretreated with a rabbit anti-DR antiserum or normal rabbit serum (NRS) plus complement as described, before being added to the cultures. The results of

Table II. *Effect of treatment of epidermal cells (Ec) with anti-DR antiserum and complement and with normal rabbit serum (NRS) and complement on the autologous T lymphocyte response to herpes simplex virus antigen (HSV-Ag) and herpes simplex live virus (HSV)*

Exp. no.	No. of Ec	Ec alone	T cells plus HSV-Ag and			T cells plus HSV and		
			Un-treated Ec	NRS-treated Ec[a]	Anti-DR treated Ec[b]	Un-treated Ec	NRS-treated Ec	Anti-DR treated Ec
1	–		527±23[c]			400±94		
	2×10^4	–	8 125±346	6 816±178	435±203	7 210±532	6 707±987	248±53
	5×10^4	56±19	13 336±831	11 171±987	522±186	9 684±1 410	9 306±411	214±88
	10×10^4	–	17 986±1 398	12 561±656	1 131±102	12 038±2 212	10 443±829	591±138
2	–		200±34			176±17		
	2×10^4	–	717±155	1 129±104	119±11	637±101	1 140±188	150±26
	5×10^4	101±14	1 574±175	1 981±216	300±29	2 180±260	1 502±198	103±29
	10×10^4	–	1 740±166	1 734±45	938±257	1 609±74	1 351±54	788±24
3	–		293±154			166±84		
	2×10^4	–	10 542±402	5 211±649	473±92	3 002±83	930±124	320±160
	5×10^4	–	14 407±780	14 142±472	415±256	3 238±152	1 951±158	548±113
	10×10^4	52±27	13 822±272	13 996±319	1 194±146	5 647±603	3 331±131	1 612±236

[a] Epidermal cells treated with normal rabbit serum and complement.
[b] Epidermal cells treated with anti-DR antiserum and complement.
[c] Mean cpm ± S.E.

three such experiments are shown in Table II. It can be seen that no, or only a weak response was obtained in cultures using epidermal cells pretreated with the anti-DR antiserum plus complement. In contrast, in cultures using epidermal cells pretreated with normal rabbit serum plus complement, the response was similar to that obtained using untreated Ec. The experiments, in which the anti-DR antiserum or NRS and complement were added directly to the wells, showed similar results.

DISCUSSION

The present study demonstrates that human epidermal cells are able to replace macrophages as regards ability to present herpes simplex virus type 1 antigen and live herpes simplex virus type 1 in an immunogeneic way to T lymphocytes. Evidence was also obtained that the cells responsible for these functions carry the HLA-DR molecules.

In a previous study we showed that the Langerhans' cells are most probably responsible for the ability of allogeneic epidermal cells to stimulate T lymphocytes (5). Furthermore, we provided evidence that they can present the antigen purified protein derivative (PPD) of tuberculin in an immunogeneic way to T lymphocytes (5). These and the present observations are in accordance with similar data obtained in studies on guinea pigs (25).

The epidermal cell suspensions used in our experiments contain three major types of cell; keratinocytes, melanocytes, and Langerhans' cells. Contamination of the cell suspensions with other types of cells from dermis is unlikely, since the epidermal sheets we used are separated form dermis at the dermo-epidermal junction (10). Absence of peroxidase-positive cells in the epidermal cell suspensions indicated that no monocytes were present.

No significant stimulation was seen in cultures containing only T cells and antigen. Maximum stimulation both with HSV-Ag as well as HSV was obtained with 5×10^3 macrophages in the cultures, i.e. a T/Mϕ ratio of 10: 1. Reduced responses were obtained when a higher number of macrophages was added. This indicates that the T/Mϕ ratio of 10: 1 gives an optimal response using our culture conditions. The response increased, however, with increasing numbers of epidermal cells added, with a maximum response obtained with 1×10^5 epidermal cells in the wells. The strongest antigen-specific responses were seen with epidermal cells (Fig. 1). Furthermore, the responses obtained with low numbers of macrophages, i.e. 1×10^3 and 5×10^3 cells, were equivalent to the responses obtained with the same numbers of epidermal cells. Since

only 3–5% of the epidermal cells were HLA–DR-positive, this may indicate that Langerhans' cells have better antigen-presenting capacity for the antigen tested when antigen was in surplus. In contrast, previous studies using PPD as antigen showed that only 3–5% of macrophages as compared with the number of epidermal cells were necessary to obtain equivalent stimulation.

The kinetics study showed a peak stimulation on day 7, for both HSV-Ag and HSV, as shown in Fig. 2. This is similar to the kinetics seen using blood-derived macrophages as antigen-presenting cells (3). It can also be seen from both Figs. 1 and 2 that the live virus usually gave lower responses than the virus antigen. A possible cytopathic effect of the live viruses on the cells in the cultures might possibly explain the difference. In some studies, the epidermal cells were pulsed with antigen. The responses obtained in these cultures were similar to the responses obtained with HSV-Ag or HSV added to the cultures. A kinetics study using the pulsed epidermal cells showed results equivalent to the cultures with antigen added, a peak stimulation on day 7.

In the cultures where we used epidermal cells pretreated with the anti-DR antiserum, no or only minimal responses were seen, while the responses in the cultures using epidermal cells pretreated with normal rabbit serum and complement were similar to the responses using untreated cells. This indicates that the HSV-Ag and HSV presenting functions were performed by HLA–DR-positive cells. Only Langerhans' cells (11, 15, 24), and the majority (85%) of the few so-called indeterminate cells in epidermis have HLA–DR antigens (16), and a relationship exists between these indeterminate cells and the Langerhans' cells. It was recently proposed that when Langerhans' cells emigrate in response to haptenic stimulation, the indeterminate cells immigrate to restore the status quo (16). Indeterminate cells may therefore well be immature Langerhans' cells.

Thus it is highly probable that it is the Langerhans' cells in the epidermal cell suspensions that are responsible for the HSV-Ag-and HSV-presenting functions.

Adherent cells may be necessary or have a potentiating effect on T lymphocyte responses to mitogens, and it is well documented that the T cell responses to specific antigens are dependent upon adherent cells (7, 8, 13, 14). Previous studies at this laboratory have shown that the adherent cell-dependent T cell responses to PPD and herpes simplex virus are restricted by the self-HLA-D/DR molecules of the T cell donor (1, 2, 4), and studies are in progress to investigate whether this is also true of the Langerhans' cell-dependent T cell responses.

In a previous study, we discussed the significance of the PPD-presenting capacity of the Langerhans' cells for the Pirquet and Moro-Hamburger tuberculin reactions (5). We concluded that it is possible that the tuberculin which is applied to the epidermis is first bound to Langerhans' cells and then presented in association with self-HLA–D/DR molecules to T lymphocytes. These events could constitute the afferent phase in the cutaneous cellular immune response to tuberculin.

The results presented here may indicate a role of the Langerhans' cells in herpes simplex skin infections. Herpetic lesions consist of grouped vesicles which develop rapidly on an erythematous base. Live virus are present in the lesions, and can be both cultured and identified by electronmicroscopy. It is possible that the herpes virus particles are bound to the Langerhans' cells and then presented in association with HLA–D/DR molecules to T lymphocytes, constituting the afferent phase in the cutaneous immune response to herpes simplex virus.

Usually, complete healing of herpes labialis takes place within 10–14 days. The absence of Langerhans' cells in cornea (26) may well be an important factor in the pathogenesis of the often persistent and chronic herpes simplex infections of corneal epithelium.

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