Role of Keratinocytes in Human Recurrent Herpetic Lesions

Ability to Present Herpes Simplex Virus Antigen and Act As Targets for T Lymphocyte Cytotoxicity In Vitro

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Abstract

In human recurrent herpetic lesions epidermal keratinocytes are induced to express HLA class II (DR) antigens. Keratinocytes derived from human split skin and cultured in vitro were induced to express HLA-DR but not -DQ antigens with IFN gamma preparations. These stimulated keratinocytes presented herpes simplex antigen directly to autologous blood-derived T lymphocytes in four of four subjects (stimulation indices: 1.5–2.7), suggesting that keratinocytes may have an accessory herpes simplex virus (HSV) antigen-presenting role in addition to the Langerhans cells and macrophages in herpetic skin lesions. Blood mononuclear cells from eight herpes simplex seropositive subjects which were activated in vitro by HSV antigen for 6 d showed cytotoxicity specific for HSV in infected autologous keratinocytes. This was significantly increased by prestimulation with IFN gamma (51–56% to 83–85%). In four of eight patients some cytotoxicity also occurred against uninfected, IFN gamma-stimulated keratinocytes. Lymphocyte subset analysis showed that cytotoxicity against HSV-infected, IFN gamma-stimulated keratinocyte targets was mediated by both CD3+ T lymphocytes and Leu 11b+ natural killer cells. T lymphocyte cytotoxicity was mediated by both CD4+ and CD8+ T lymphocytes, suggesting a cytotoxic role for the activated CD4+ lymphocytes that initially predominate in herpetic lesions.

Introduction

The humoral and cellular immune responses to primary herpes simplex infection have been extensively studied in rodents and humans. Animal models have been particularly useful in primary infection but they have not been as close an approximation to human recurrent disease (summarized in reference 1). The predominant immune cell types in recurrent herpes simplex lesions were recently described (2). Serial biopsies of lesions from 0.5 to 6 d after onset demonstrated early infiltration of CD4 positive T lymphocytes and Leu M3 positive monocyte/macrophages into subepidermal and perivascular regions of the dermis. The normalization of CD4/CD8 lymphocyte ratios in skin after 2–3 d suggested a later influx of CD8 positive T lymphocytes. Leu 11+ natural killer cells were underrepresented in the dermal infiltrates (1–4%) and B lymphocytes were rare. Epidermal keratinocytes within and adjacent to the papule, vesicle or crust expressed HLA-DR antigens and this became uniform within 2 d. IFN-gamma, which has been detected within these lesions (2, 3), and other lymphokines secreted by T lymphocytes and monocytes probably induced keratinocyte DR expression (4, 5). The close proximity of Herpes simplex virus (HSV)[1]–infected keratinocytes and T lymphocytes within the subjacent infiltrate strongly suggested immunologic interactions: HSV-infected DR positive keratinocytes might present antigens to CD4 positive T lymphocytes or act as targets for cytotoxicity mediated by CD4+ or CD8+ T lymphocytes. We have developed an in vitro model in which the human keratinocyte expressing HLA-DR induced by interferon gamma (and perhaps other lymphokines) can subserve both of these functions.

Methods

Patients. Normal HSV seropositive patients undergoing split skin grafts for minor surgical procedures were selected as donors of split skin for keratinocyte culture and of autologous blood mononuclear cells.

Keratinocyte cultures. Split skin was transferred into medium containing high concentrations of antibiotics (Dulbecco's MEM, 0.5% FCS and pencillin, streptomycin, gentamicin, and amphotericin B) and then cut into 0.5-cm squares. The pieces were rinsed successively in PBS 70% alcohol, and low antibiotic medium (Dulbecco's MEM, 0.5% FCS and penicillin, streptomycin, gentamicin, and amphotericin B), incubated in 0.01% trypsin overnight and then diced into 1-mm squares. The fragments were allowed to adhere to T25 tissue culture flasks (Falcon Labware, Oxnard, CA) and growth medium (Eagle's MEM, with 10% FCS, (Flow Laboratories, McLean, VA) 10-mM Hepes, 10 ng/ml epidermal growth factor, cholera toxin 10^{-10} M and 0.5 μg/ml hydrocortisone was added (6).

After 4–7 d, outgrowths of epidermal keratinocytes from the explants were noted by phase-contrast microscopy and these slowly became confluent as a monolayer over 2–4 wk. Medium was changed twice weekly. Explants with obvious fibroblast outgrowths were removed mechanically. To disperse the monolayer into a single cell suspension, it was first washed with 0.2% EDTA to remove residual fibroblasts and then incubated with 0.25% trypsin/EDTA at 37°C. The washed suspension was dispensed into microtiter wells at a cell concentration of $1–2 \times 10^4$ per well. Contaminating fibroblasts as detected by immunoperoxidase staining for vimentin with anti–vimentin MAb (DAKOPATTS, Copenhagen, Denmark) were < 2% in all patients' cultures (7). This low proportion of contaminating fibroblasts was supported by staining for keratins with rabbit polyclonal antisera (DAKOPATTS). 88–92% of plated cells were keratin positive, 5–8% were large keratin-negative cells, probably basal cells, and 2–4% were small keratin-negative cells, probably mostly fibroblasts. In microtiter wells, > 95% of the cells had morphologic features of keratinocytes and in some wells held for 2 wk, no fibroblast overgrowths were noted. For

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1. *Abbreviations used in this paper:* HSV, herpes simplex virus; $TCID_{50}$, 50% tissue culture infective dose.

examination by direct immunofluorescence keratinocyte cultures were grown in slide chambers (Lux; Flow Laboratories).

MAb. Anti-Leu 3a (CD4), -Leu 2 (CD8), -Leu 4 (CD3), -Leu 11b (CD16), -Leu 10 (DQ) and anti-HLA-DR were obtained from Becton-Dickinson & Co., Immunocytometry Systems, Mountain View, CA. Anti-OKT4, -OKT8, and -OKT3 were obtained from Ortho Diagnostics, Raritan, NJ.

Preparation of mononuclear cells and lymphocyte subsets. Mononuclear cells were separated from 40–100 ml of heparinized blood over a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, NJ). Purified T lymphocytes for the antigen presentation studies were obtained by passage of mononuclear cells over a nylon wool column as previously described (8). The proportion of contaminating monocyte/macrophages as determined by staining cytocentrifuged smears for nonspecific esterase (9) was < 0.5%. Mononuclear cells were depleted of CD4+ and CD8+ T lymphocytes and Leu 11+ natural killer cells for cytotoxicity studies by complement-dependent cytotoxicity using anti-OKT3, anti-OKT4, anti-OKT8 (Ortho Diagnostics), anti-Leu M3, and anti-Leu 11b (Becton-Dickinson & Co.) MAb as previously described. (8). Briefly, 10^7 mononuclear cells were incubated with 0.1 μg of MAb at 0°C for 30 min, resuspended, washed, and reincubated with prediluted baby rabbit complement (Pel-Freez) for 45 min. The extent of cytotoxicity was assessed by staining with 1:10 trypan blue and counting in a hemocytometer. The viable cell population was recovered after three washes. Residual contaminating cells were < 1% as determined by flow cytometry using an MAb directed against a different epitope of the same subset marker (e.g. anti-Leu 3a for cytotoxicity with anti-OKT4) or goat anti-mouse IgG to detect residual antibody on unkilled cells and flow cytometry.

Subset deletion was chosen as the method of choice for assessing the function of a subset, thus avoiding functional inhibition by antibody adherent to CD4 or CD8 proteins (10, 11).

Virus and antigen. The F strain or a freshly isolated wild strain of Herpes simplex type 1 were used for infection of keratinocytes (12) and preparation of heat inactivated antigen from infected cell lysates as previously described (8). Briefly, antigen was prepared by heating supernatants of freeze-thawed and sonicated HSV-infected Hep2 cells (10^6 50% tissue culture infective dose ($TCID_{50}$)/ml) to 56°C for 30 min. Control antigen was prepared by omitting HSV.

Lymphokine preparations. Recombinant IFN gamma (specific activity = 2×10^7 U/mg protein; Genentech, South San Francisco, CA), purified IFN gamma (specific activity = 10^6 U/mg; Cellular Products, Buffalo, NY), and supernatants from PHA-stimulated mononuclear cells (PHA supernatants) were used to stimulate keratinocytes. IFN gamma concentrations in all preparations were assayed and standardized by a dual MAb RIA (Centocor, Malvern, PA) (13).

Measurement of Herpes simplex antigen specific T lymphocyte activation. Herpes simplex antigen was added to mixed keratinocyte-T lymphocyte cultures (ratio 1:15 or 1:30) in microtiter wells at the optimum dilution (11). After 4 d incubation at 37°C, 1 μCi/well [^{3}H]-thymidine was added and the cells harvested 18 h later with a cell harvester (Titertek; Flow Laboratories, McLean, VA). Incorporated radioactivity was read in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). HSV-specific T lymphocyte activation was calculated as: stimulation index = HSV-specific T lymphocyte activation (cpm/well)/nonspecific T lymphocyte activation (cpm/well).

Assay of cytotoxicity against keratinocyte targets (modified from 14, 15). Keratinocytes were inoculated with Herpes simplex virus (10^5 $TCID_{50}$/microtiter well) for 1 h, washed twice with RPMI 1640 medium containing 10% FCS, incubated with ^{51}Cr (10 μCi/well) for 90 min, and washed four times with RPMI 1640/10% FCS. Effector mononuclear cells, stimulated with control or Herpes simplex antigen for 6 d in RPMI 1640/20% human serum (seronegative for HSV), were added at effector target ratios ranging from 20:1 to 80:1 and incubated for 18 h. Supernatant radioactivity was detected in a gamma counter (model 1185; G. D. Searle & Co., Skokie, IL) and % cytotoxicity calculated as: % specific ^{51}Cr release = test ^{51}Cr release − spontaneous ^{51}Cr release/total ^{51}Cr release − spontaneous ^{51}Cr release.

Preliminary cytotoxicity assays. In preliminary cytotoxicity experiments the following observations were made. (*a*) Increased washing of keratinocytes was required to reduce the initially high levels of spontaneous release of ^{51}Cr. (*b*) Keratinocytes were often resistant to detergent lysis and also needed freeze-thawing to liberate total ^{51}Cr. (*c*) IFN gamma preparations reduced ^{51}Cr uptake and release (similar to its effect on [^{3}H]thymidine), hence reducing both spontaneous and total ^{51}Cr release variably by 23–70% according to the experiment. (*d*) The effects of PHA supernatants and purified IFN gamma on ^{51}Cr uptake and on cytotoxicity were similar (see Table II). (*e*) Effector-target ratios of 80:1 were much superior to 20:1 (Table II). Preliminary experiments comparing ratios of 100:1 and 50:1 to 80:1 demonstrated the peak in percentage specific ^{51}Cr release was achieved at the 80:1 ratio and no further increase occurred at 100:1. This ratio was used throughout these experiments to minimize use of donor PBMC.

Statistics. Differences between the sets of results (expressed as mean counts per minute/well of released ^{51}Cr) which were obtained with different treatments were assessed for statistical significance by *t* test, with modifications of the degrees of freedom to allow for unequal variances.

Results

Lymphokine induction of HLA-DR expression by keratinocytes. Human keratinocytes (2×10^5/cluster well) were incubated with recombinant IFN gamma, purified IFN gamma, and PHA supernatants at concentrations of 20 to 100 U/ml for 4 d. The cells were detached with 0.25% trypsin/0.2% EDTA and stained with anti-HLA-DR and -DQ (anti-Leu 10) MAb and examined by flow cytometry (10,000 cells counted). All preparations induced 85–90% of keratinocytes to express HLA-DR but not -DQ at optimal concentrations of 50–100 U/ml, (Fig. 1), consistent with previously reported results (4, 5). No Leu 6+, HLA DR+ Langerhans cells, or HLA DR+ dermal dendritic cells were detected in parallel control keratinocyte cultures.

Herpes simplex antigen presentation by IFN gamma-stimulated keratinocytes to nylon wool purified T lymphocytes. 1/10 dilutions of HSV antigen (previously determined to be optimal for antigen presentation) were added to keratinocytes ($1–2 \times 10^4$/well) stimulated with either recombinant or purified IFN gamma or PHA supernatants (100 U/ml) for 4 d and mixed with nylon wool–purified lymphocytes (3×10^5/well). T lymphocyte activation was determined by [^{3}H]thymidine incorporation. Preliminary experiments showed that addition of [^{3}H]thymidine for 18 h at day four produced higher incor-

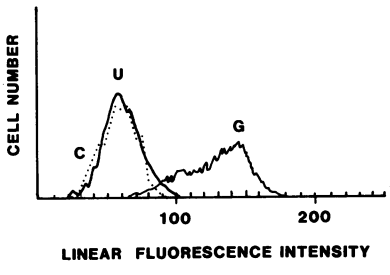

Figure 1. HLA-DR expression by human epidermal keratinocytes before and after stimulation with IFN gamma. Human keratinocytes (2×10^5/cluster well were stimulated with 100 U/ml recombinant IFN gamma for 4 d, detached with trypsin-versene, stained with fluorescein-conjugated anti-HLA-DR or control MAb (anti-Leu 2b) and examined by flow cytometry. Mock-stimulated keratinocytes were also stained with anti-HLA-DR MAb. U, unstimulated + anti HLA-DR MAb; C, IFN gamma + control MAb; and G, IFN gamma + anti HLA-DR MAb. Note the high degree of autofluorescence of keratinocytes not expressing HLA-DR.

poration than addition at days 3 or 5 (e.g. day 3, 3,654±178; day 4, 4,538±240; day 5: 4,050±289; mean±1SD cpm). Controls for nonspecific lymphocyte activation were obtained by using similar cultures but omitting IFN gamma, HSV antigen, IFN gamma and keratinocytes, or incubating NWP lymphocytes alone. All test and control experiments were performed in triplicate.

As shown in Table I, the significant increase in [^{3}H]-thymidine incorporation by NWP cells after HSV antigen stimulation in patient 1 suggested contamination with a small proportion of antigen-presenting cells. Similarly, in the two cultures tested, IFN gamma-stimulated (and HLA-DR expressing) keratinocytes induced a small but significant increase in T lymphocyte activation, in the absence of HSV antigen. Addition of HSV antigen to the latter cultures produced a marked and significant increase in T lymphocyte response in all four cultures ($P < 0.02$, $t = 4.59$–14.15; stimulation indices 1.5–2.7; Table I). Nevertheless, this maximum response was less than observed in HSV antigen–stimulated PBMC or macrophage-T lymphocyte cultures (10). Recombinant IFN gamma-stimulated keratinocytes did not induce T lymphocyte activation with or without HSV antigen (patients 3 and 4). Incubation of neutralizing MAb to IFN gamma (B3; kindly supplied by Dr. J. Vilcek; reference 15) with PHA supernatants for 1 h before addition of the mixture blocked the T lymphocyte response to keratinocytes without HSV antigen and also partially blocked the antigen-induced response (patient 4).

Cytotoxicity of HSV antigen–stimulated mononuclear cells for keratinocytes with and without IFN gamma stimulation. In preliminary experiments HSV at 10^5 $TCID_{50}$/ml was added to keratinocytes with and without prior stimulation with PHA supernatants (100 U/ml of IFN gamma) in slide chambers. The cells were fixed with acetone at 8, 18, and 24 h and stained with fluorescein-conjugated MAb to HSV glycoprotein C. By 8 h, intense cytoplasmic fluorescence was observed in most unstimulated keratinocytes. At this time, occasional stimulated keratinocytes showed focal cytoplasmic fluorescence but equivalent diffuse cytoplasmic fluorescence was only observed at 18 h. Cytopathic effect was obvious in unstimulated keratinocytes at 18 h but was less marked in stimulated keratinocytes (reference 11). Similarly, ^{51}Cr release from HSV-infected keratinocytes was not significantly raised above spontaneous release levels in four cytotoxicity experiments.

Table II shows the HSV-specific and control cytotoxicity assays for eight subjects. There was a wide variation in ^{51}Cr uptake and hence total and spontaneous release. Mononuclear cells that had not been activated with HSV antigen showed no significant cytotoxicity (in comparison with spontaneous release). However, in four of eight of the subjects (patients 2, 5, 6, 7 and also patient 2 [Table III], where target keratinocytes were not infected) small but significant increases in cytotoxicity against uninfected but IFN gamma-stimulated target keratinocytes was observed. (An even smaller degree of cytotoxicity, 15% specific ^{51}Cr release, was seen in one of three experiments with uninfected, unstimulated keratinocytes.) The cell types involved in this autoreactive cytotoxicity are analyzed in Table III. Both T lymphocytes and Leu 11b+ natural killer cells were active, with the former predominating. T lymphocyte cytotoxicity against these IFN-stimulated HLA-class I and II antigen-expressing cells was reduced approximately equally by deletion of CD4+ and CD8+ lymphocyte subsets.

Mononuclear cell cytotoxicity specific for HSV-infected keratinocytes was augmented by IFN gamma stimulation of the keratinocytes (Table II) as shown by comparison of percent specific ^{51}Cr release with and without IFN gamma stimulation of keratinocytes in patients 1, 2, and 5 i.e., 83 vs. 56%, 86 vs. 51%, and 82 vs. 17%, respectively. In patient 1, there was no autoreactive cytotoxicity and still a difference of 27%. However, the large difference in patient 5 may have been partly due to extensive autoreactive cytotoxicity contributing to apparent HSV-specific class II restricted cytotoxicity (compare also in Tables III and IV). Mononuclear cell cytotoxicity for HSV-infected, IFN gamma-stimulated keratinocytes was significantly greater than that for uninfected target keratinocytes in all nine experiments (eight patients) shown in Table II and ranged from 43 to 86% of specific ^{51}Cr release. Attempts were made to determine the lymphocyte types mediating the cytotoxicity initially by panning i.e., by adherence of specific lymphocyte subsets to a subset-specific MAb adherent to a solid phase (8).

Table I. Herpes Simplex Antigen Specific T Lymphocyte Activation by IFN Gamma-stimulated Keratinocytes

Patient no.	[^{3}H]Thymidine incorporation					
	T lymphocytes alone	Herpes antigen + T lymphocytes	Keratinocytes + T lymphocytes	IFN γ-stimulated* keratinocytes + T lymphocytes‡	Herpes antigen + keratinocytes + T lymphocytes	Herpes antigen + IFN γ-stimulated keratinocytes + T lymphocytes‡
	mean cpm/microtiter well ± 1 SD					
1	772±232	2,442±353	ND	2,283±238	2,750±289	6,109±948
2	1,147±215	1,921±353	ND	1,858±242	2,192±266	4,552±1,041 (2,006±248)§
3	2,266±590	ND	2,393±157	3,190±112	2,998±221 (3,057±276)§	4,781±195 (3,760±515)§
4	1,668±307	ND	1,281±321	2,582±226‖ (1,050±104)	1,410±337	5,616±1,030‖ (1,025±282)§

* IFN$_\gamma$ = supernatants from PHA-stimulated mononuclear cells. ‡ Stimulation index = HSV-specific T lymphocyte activation/non-specific T lymphocyte activation = 2.7, 2.3, 1.5, and 2.2 for patients 1, 2, 3, and 4, respectively. $t = 7.56$, 5.42, 14.15, and 5.75 for patients 1, 2, 3, and 4, respectively; $P < 0.01$, 0.02, 0.001, 0.01. § Stimulated with recombinant IFN gamma. ‖ After blocking PHA supernatant stimulation of keratinocytes with B3 anti-IFN gamma MAb: 2,582±226 → 1,092±141; 5,616±1,030 → 3,354±178.

Table II. Cytotoxicity of HSV Antigen-stimulated Mononuclear Cells for IFN Gamma-stimulated and HSV-infected Keratinocytes

Patient	Lymphokine preparation	Controls		Test	
		No HSV antigen stimulation	No HSV infection (of keratinocytes)	20:1	80:1
		Mean percentage specific ^{51}Cr release (±1 SD)			
1	PHA S/N	4.3±5.8	13.4±4.7	14.5±2.2	83.5±4.1*
	(No PHA S/N)				(56.0±20.2)
	pIFN-γ	ND	6.8±8.7	5.5±2.2	64.4±3.2*
2	PHA S/N	15.1±1.3	28.7±7.1		85.6±18.0*
	(No PHA S/N)				(50.7±4.1)
3	PHA S/N	7.9±4.4	11.4±7.9		42.7±10.5*
4	PHA S/N	−12.4±7.8	9.9±13.0		46.0±12.8*
5	PHA S/N	10.1±4.2	72.2±6.0		82.0±2.1‡
	(No PHA S/N)				(16.5±2.8)
6	PHA S/N	ND	32.4±1.7		71.6±8.2*
7	pIFNγ	24.0±5.7	56.5±4.9		82.1±2.5*
8	IFN S/N	−2.3±2.0	−1.2±3.3		51.1±3.8*

All cytotoxicity assays shown used an E/T ratio of 80:1. Spontaneous release values fell within the range of 20 to 30% of total releasable counts of ^{51}Cr. * $P < 0.001$, ‡ $P < 0.05$ by t test for differences in mean counts per minute/well of released ^{51}Cr between infected and uninfected keratinocytes.

Poor separation was achieved with the mononuclear cells that had been stimulated with HSV antigen for 6 d. However, subset deletion using anti-OKT3, -OKT4, -OKT8, and -Leu 11b and complement was satisfactory, with < 1% residual contaminating cells as determined by flow cytometry. Treatment with complement alone in two patients did not significantly reduce mononuclear cell cytotoxicity (data not shown). As shown in Table IV, both T lymphocytes and Leu 11b natural killer cells mediated the cytotoxicity. In six of seven experiments focusing on the T lymphocyte cytotoxicity, there was no significant difference between CD4+ and CD8+ lymphocyte-mediated killing, but in one experiment (patient 1) CD4+ T lymphocyte killing predominated.

Discussion

In this in vitro model of a human recurrent herpes simplex lesion, IFN gamma or mixed lymphokine-stimulated, HLA-DR expressing human keratinocytes were capable of both presenting herpes simplex antigen to T lymphocytes and acting as targets for HSV-specific T lymphocyte cytotoxicity. Lymphocyte subset analysis also showed that CD4+ lymphocytes were capable of cytotoxicity against these HSV-infected DR+ keratinocytes.

Table III. Mononuclear Cell Cytotoxicity for Uninfected IFN Gamma-stimulated Keratinocytes: Results of Lymphocyte Subset Deletion

Patient	Untreated	-CD3	-CD4	-CD8	-Leu 11b	-Leu M5
	Mean percentage specific ^{51}Cr release (±1 SD)					
1	72.2±6.0	27.4±5.6	57.6±2.5	53.2±3.7	56.9±6.3	
2	37.7±14.9*	9.0±4.4	ND	ND	19.7±2.6*	33.8±16.5*
3	32.4±1.7‡	8.9±4.3	24.5±8.0‡	23.5±4.8	ND	

All cytotoxicity assays shown used an E/T ratio of 80:1. Ratios of 20:1 showed no significant percent specific ^{51}Cr release. Spontaneous release values fell within the range of 20 to 30% of total releasable counts of ^{51}Cr.
*‡ Differences not significant by t test.

It is now well established that IFN gamma can induce HLA-class II antigen expression de novo by a wide variety of cell types (17). However, these cells vary in their ability to present antigen, even when class II antigen expression is equivalent (18). In the experiments described here, the antigen-presenting capacity of the DR+ keratinocytes was relatively weak compared with macrophages or monocytes (8, 11), even in the presence of IL 1 (data not shown). This requires further investigation, but qualitative differences linked with the lack of expression of DQ or quantitative differences such as a relatively lower density of membrane DR expression or the absence of an important costimulatory signal may all be important. In this respect, the relatively poor induction of antigen-presenting capacity by recombinant IFN gamma compared with PHA supernatants or partially purified IFN gamma (Table I) seems paradoxical as all were standardized for IFN gamma content and were approximately equal in inducing HLA-DR expression. This also requires further study but may be explained by lower stability of the recombinant preparation or the presence of other important HLA class II antigen-stimulating lymphokines, such as tumor necrosis factor, in the less purified preparations (19). In the keratinocyte-autologous T lymphocyte system described here it is important to maintain fibroblast contamination at a very low level, as IFN gamma-containing preparations can also induce HLA-DR expression on these cells, although their antigen-presenting capacity is very weak (18). The importance of Ia+, Leu 6+ Langerhan's cells as antigen presenting cells in skin has been well established (15, 20) but these cells were not detected in this system. However, it seems likely that in vivo HSV-infected keratinocytes would provide only accessory HSV antigen presentation to that provided by Langerhan's cells and dermal dendritic

Table IV. Mononuclear Cell Cytotoxicity Specific for HSV-infected, IFN Gamma-stimulated Keratinocytes Results of Lymphocyte Subset Deletion (by MAb and Complement)

Patient	Lymphokine preparation	Untreated	-CD3	-CD4	-CD8	-LEU 11b
		Mean percentage specific ^{51}Cr release (±SD)				
1a	PHA S/N	54.7±9.1*	ND	36.0±7.1	47.2±3.7*	49.6±2.9*
b	Purified IFN γ	85.2±17.1	ND	54.4±6.8	50.9±8.0	45.1±3.8
2a	PHA S/N	55.8±1.8‡	22.3±2.5	28.7±5.31	38.6±20.7‡	ND
b	Purified IFN γ	59.2±2.2	35.2±3.0	40.3±2.5	46.2±4.1	ND
3	PHA S/N	82.0±2.1§	27.4±5.6	57.6±2.5	53.2±3.7	56.9±6.3
4	PHA S/N	71.6±8.3	40.1±4.2	43.8±4.9	39.0±7.4	ND
5	Purified IFN γ	82.1±2.5	50.1±7.9	51.2±2.0	53.3±8.4	ND

All cytotoxicity assays shown used an E/T ratio of 80:1. Ratios of 20:1 showed no significant percent specific ^{51}Cr release. Spontaneous release values fell within the range of 20 to 30% of total releasable counts of ^{51}Cr. *‡ Differences not significant by *t* test. § Represents HSV-specific and nonspecific killing.

cells, present in skin at the commencement of HSV reinfection, and infiltrating activated macrophages, capable of digesting whole virus into small peptides. The latter cells have the advantage of constitutive HLA class II antigen expression, whereas induction of these antigens on keratinocytes by lymphokines secreted by activated T lymphocytes requires 1–2 d (2, 4).

HSV antigen–primed mononuclear cell cytotoxicity for HSV-infected keratinocytes was greater when these cells were stimulated with IFN gamma and expressed HLA-DR in vitro, suggesting that augmentation of class I restricted cytotoxicity and recruitment of class II restricted CD4+ lymphocyte-mediated cytotoxicity by this mechanism are important in vivo. The in vitro results were similar whether partially purified IFN gamma or PHA supernatants were used for stimulation.

The variable mononuclear cell cytotoxicity for uninfected IFN gamma-stimulated keratinocytes, mediated by CD4+ and CD8+ T lymphocytes and Leu 11+ natural killer cells, partially obscured HSV specific cytotoxicity to different degrees in 50% of the patients. Similar autoreactivity was noted in the antigen presentation experiments. In both cases it was more prominent when keratinocytes were stimulated with IFN gamma, inducing HLA class II antigen expression and increasing the intensity of HLA class I expression. Similar results have also been reported in which CD4+ lymphocyte mediated cytotoxicity was found to be specific for components of FCS present in the culture medium and taken up by target cells (21). In our experiments, human serum was used in culture medium for HSV antigen priming of T lymphocytes and the keratinocytes were cultured in FCS. Possibly the cytotoxic T cells may have recognized antigenic epitopes common to both sera or other endogenous or exogenous antigens expressed on the surface of keratinocytes in association with HLA-DR. The variability observed may relate to differences in the concentrations of residual antigens, to differential affinity of serum antigens for different HLA-DR phenotypes or to augmented induction of autoreactivity by certain phenotypes. Recently Tilkin et al. have demonstrated that in vitro stimulation with (influenza) viral antigens induced both virus-specific and autoreactive T cell clones (22). The small number of autoreactive clones were specific for HLA-DR antigens and were capable of both proliferative and cytotoxic activity, very similar to the results obtained here. Recent crystallographic studies of HLA class I antigens have also shown that peptide antigens, probably derived from the medium or the cell itself, are always bound to the HLA molecule providing appropriate autoreactive stimuli or targets (23). Culture of keratinocytes in defined serum-free medium should determine whether serum antigens are important (24). If the cytotoxicity was directed against residual endogenous or exogenous antigens it raises the question of whether autoreactive cytotoxic T cells directed against antigens associated with newly induced HLA class II or denser class I antigens might restrict the spread of HSV infection by killing adjacent keratinocytes before membrane expression of HSV antigens. By analogy, epidermal damage, manifested by lichenoid tissue reaction, has been induced by injection of murine self Ia-specific autoreactive cytotoxic T cells into the footpads of mice (25). Presumably natural killer cells activated by HSV glycoproteins or by IFN and IL 2 within the primed cultures in vitro showed some slight reactivity towards cell membrane antigens altered by interferon gamma stimulation (26–28).

The importance of CD8+ lymphocyte-mediated, HLA class I antigen-restricted cytotoxicity, natural killer cell activity, and macrophage activation in immunity against primary HSV infection has been well established in animal models (29–31). Although CD4+ lymphocyte-mediated class II-restricted cytotoxicity has also been well demonstrated in vitro against influenza and by clonal studies against HSV and measles (32–34), there is skepticism about the importance of their role in vivo (35). Further evidence for its importance in herpesvirus infections has been demonstrated recently with EBV and HSV infection (36, 37). In the latter study of HSV1 seropositive patients blood cytotoxic precursor frequency to HSV-infected, EBV-transformed B cells was determined. CD4+ lymphocyte mediated, class II-restricted cytotoxicity and natural killer cell activity predominated, similar to the results reported here with keratinocyte targets. The experiments in Table I show clear evidence of approximately equal CD4+ and CD8+ T lymphocyte cytotoxicity and natural killer cell activity specific for HSV-infected, IFN gamma-stimulated keratinocytes. These results are predictable from current models of cytotoxicity. IFN gamma not only induces membrane expression of HLA class II antigens in many cell

types but also increases class I antigen density (4, 5). CD8+ cytotoxic T lymphocytes recognize alloantigens in association with HLA class I antigens and CD4+ cytotoxic T lymphocytes usually in association with HLA class II (34, 37). The mode of induction of CD4+ cytotoxic T lymphocytes by HSV antigen presentation by monocytes macrophages and dendritic cells in vitro probably also occurs in vivo as macrophages make up 10–15% of mononuclear infiltrate cells in herpetic lesions and Langerhans cells and probably dermal dendritic cells are present (2). The generation of lymphokines within the HSV antigen–primed mononuclear cultures or direct stimulation by HSV glycoproteins would be expected to activate natural killer cells, which have been previously shown to kill HSV-infected fibroblasts (26–28).

Although this keratinocyte-autologous T lymphocyte model has been most useful in confirming the suggested immunologic role of the HLA-DR+ keratinocyte in recurrent herpetic lesions, it needs to be refined, particularly if it is to be used in evaluating structural and nonstructural proteins that are targets for HSV-specific cytotoxicity in the lesions (39). The most important of these is the limitation on numbers of keratinocytes and autologous T lymphocytes that can be easily obtained, thus restricting the scope of comparative experiments in the one patient. It is possible that HSV antigen priming of mononuclear cells (using HLA-DR and -DQ expressing monocytes/dendritic cells for antigen presentation) may lead to differences in antigenic epitope recognition on HLA-DR+ keratinocytes, although this probably occurs in the lesion. Increasing the supply of keratinocytes, perhaps by transformation with SV40-Adenovirus 12 recombinant virus (40), would allow comparisons of keratinocytes and monocytes as antigen-presenting cells in the priming reaction. The immunologic and biologic changes induced by transformation would have to be carefully compared with control keratinocytes. The natural killer cell activity demonstrated here is unlikely to be a prominent feature in vivo as the cells are underrepresented in recurrent herpetic lesions compared with peripheral blood (2). Interference with HSV specific cytotoxicity by autoreactive cytotoxicity, marked in one of eight patients, limits the reproducibility and clarity of the assay for assessment of important HSV target peptides in this model. Cloning of HSV-specific CD4+ and CD8+ cytotoxic T cells or estimating the specific cytotoxic precursor frequency to the various peptides may eliminate this problem, (39, 41) avoid irrelevant natural killer cell activity, and determine the target peptides for class I- or class II-restricted cytotoxicity. In view of the predominance of CD4+ T lymphocytes in lesions at the time when HSV titers in vesicle fluid start to fall (2, 42), they may be the most important cytotoxic cell involved in limiting local spread of HSV infection and subsequent eradication of epidermal infection. This hypothesis is consistent with the marked persistence of anal recurrent herpetic lesions in patients with AIDS (43).

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