

# Evolution of Recurrent Herpes Simplex Lesions

## An Immunohistologic Study

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### Abstract

We performed immunoperoxidase stains on skin biopsies taken from nine patients with recurrent peripheral herpes simplex lesions at 12 h to 6 d after onset of signs or symptoms to phenotype the inflammatory infiltrate, to detect cells producing interferons alpha and gamma, and to locate herpes simplex virus antigen-containing cells. Viral glycoprotein antigen was located in the nuclei and cytoplasm of necrotic epidermal cells, often within vesicles, in biopsies taken between the first and third day. Histologically, biopsies of all stages showed intra-dermal focal perivascular and diffuse mononuclear inflammatory infiltrates. The cells constituting the infiltrates were predominantly T lymphocytes with lesser numbers of histiocytes; Leu 7<sup>+</sup> (most natural killer/killer) cells and B cells were rare in the biopsy specimens. Leu 3a<sup>+</sup> ("helper") T lymphocytes predominated in both subepidermal and perivascular regions of early lesions (12–24 h). Tissue helper/suppressor ratios ranged from 6.3 to 3.4 compared with 1.9–1.0 in blood. In later lesions (after 2 d), monocytes/macrophages were more prominent in tissue sections and the helper/suppressor ratios (2.3–2.5) more nearly approximated those of blood (1.6–2.7). The negative correlation of tissue ratios with time was significant ( $P \leq 0.02$ ). A large proportion of the infiltrated T lymphocytes expressed DR antigens. There was also diffuse strong DR expression on epidermal cells in five cases (all at two or more days). In six biopsies, scattered macrophages and small cells, presumably lymphocytes, demonstrated cytoplasmic or membrane staining for a substance which copurifies with interferon gamma. We identified such stained cells within vessels, suggesting that these cells circulate. Gamma interferon might have an important role within the herpetic lesion, possibly inducing macrophage activation and cytotoxic T lymphocytes and increasing DR expression on monocytes and epidermal cells.

### Introduction

Immune defense mechanisms against primary and recurrent herpes simplex viral (HSV)<sup>1</sup> infection have been intensively

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1. *Abbreviations used in this paper:* HSV, herpes simplex virus; K, killer cell; NK, natural killer cell; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

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investigated in vitro and in animal models over the past two decades. Although many mechanisms involving a variety of cell types, antibody, complement, lymphokines, and interferon have been demonstrated, their relative importance and interaction in restriction of HSV replication remains unsettled. Some evidence in murine systems suggests immunologically nonspecific mechanisms, such as macrophage activation, natural killer (NK) cell activity, and interferon alpha or beta production, may be important early in primary infection. Alternatively, specific immune functions such as cytotoxic T lymphocytes, antibody-dependent cell-mediated cytotoxicity, antibody- and complement-dependent virus neutralization, and lysis of infected cells may be of greater importance in late primary and recurrent infection (1). Experiments in mice have also suggested cell-mediated immunity may play a role in maintenance of HSV latency (2). Less is known about the relative roles of these mechanisms in man (3). Recently, we reported that the titer of interferon gamma produced spontaneously by peripheral blood mononuclear cells (PBMC) of patients with recent recurrent herpes labialis appeared to predict the time to next recurrence (4). The spontaneous interferon was produced by circulating Leu 3<sup>+</sup> T lymphocytes (5). We believe that these cells may be activated in the local lesion or draining lymph node and then circulate. Hence, the present study was undertaken to phenotype the infiltrate within these lesions (6) and correlate the proportions of cells present with PBMC, to identify cells producing interferons alpha and gamma, and to determine the cellular location of HSV antigen. The immunophenotyping technique has recently provided interesting insights into the immunopathogenesis of leprosy and multiple sclerosis (7, 8).

### Methods

Nine consecutive adult volunteers with recurrent peripheral herpes simplex infection (not perioral or genital) were studied after our protocol was approved by the Stanford University Committee on Human Investigation. Clinical features were assessed by two of us (Dr. Cunningham and Dr. Miller) and the lesions staged as erythematous papules, vesicles, ulcers, or crusts. Following infiltration with local anesthetic, two biopsies were taken from each lesion either immediately adjacent (two patients) to or including 25–50% of the vesicle or crust (with the vesicle or crust margin in the center of the biopsy) using a 2-mm disposable Baker cutaneous punch (Baker Co., Inc., Sanford, ME). One specimen was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin. The other specimen was snap frozen and stored at  $-50^{\circ}\text{C}$  for later immunoperoxidase staining. The vesicles opened by the biopsies were swabbed and cultured for herpes simplex virus in human foreskin fibroblast cultures. Two blood samples were obtained for separation of mononuclear cells with Ficoll-hypaque gradients: one at the time of biopsy for cytofluorographic quantitation of lymphocyte subsets and monocytes and estimation of (spontaneous) interferon production and a second specimen was also taken for estimation of spontaneous interferon production 10 d later.

**Immunoperoxidase staining for frozen sections.** All biopsies were oriented so that sections were cut serially from the central part perpendicular to the vesicle margin. Cryostat sections, 4  $\mu$ m thick, were cut from the frozen block, fixed in acetone, and stained by an immunoperoxidase technique. Staining employed sequential incubations with murine anti-human monoclonal antibody, goat anti-mouse IgG, and swine anti-goat IgG peroxidase conjugate (Tago Inc., Burlingame, CA) with intervening phosphate-buffered saline (PBS) washes. Diaminobenzidine was the chromogen, with copper sulphate darkener and methylene blue counterstain. A peroxidase-conjugated swine anti-rabbit IgG (Tago Inc.) was substituted in two-stage staining with rabbit antiserum. All cells staining for Leu 2, Leu 3, Leu 4, Leu 7, and Leu M3 in the infiltrates of each serial section were counted without knowledge of the stage of the lesion. The proportions of peroxidase positive to total number of cells in the infiltrate were compared with the proportions in PBMC. Leu 3<sup>+</sup> macrophages were omitted from the counts of Leu 3<sup>+</sup> cells (9).

**Antibodies used for staining tissue and PBMC.** Monoclonal antibody specificities for immunophenotyping of PBMC and cellular infiltrates in tissue sections and their sources are shown in Table I. All antibody preparations were being concurrently used for immunophenotyping of skin and lymph nodes in the immunopathology laboratory and all were active. Monoclonal antibodies crossreactive to glycoproteins A/B and D (type common) of both types I and II HSV were developed by one of us (Dr. Para) and used for detection of HSV glycoprotein antigens in infected cells (18). Antigen and type specificity of the antibodies was determined by assessing the electrophoretic mobilities of labeled HSV<sub>1</sub> and HSV<sub>2</sub> proteins precipitated by them. High-titered rabbit polyclonal antibodies to interferon alpha (antisera neutralizing  $1.8 \times 10^4$  units of leukocyte interferon/0.5 ml) and gamma interferon (antisera neutralizing 20,000 units of native interferon gamma/0.5 ml) were kindly provided by Sydney Pestka (Nutley, NJ) and Jan Vilcek (New York), respectively (20, 21). Purified recombinant interferon alpha A was the immunogen for preparation of the antibody to interferon alpha. After a three-step purification process (21), the interferon gamma used to prepare the latter antibody was virtually free of interleukin 1 or 2 but was still contaminated with some lymphotoxin and human IgG. The capacity of antibodies to interferons alpha and gamma to stain smears of unstimulated PBMC or PBMC stimulated with HSV antigen for 2 d (known to induce mainly alpha interferon) or phytohemagglutinin (PHA) for 4 d (for gamma interferon

induction) was examined by indirect immunofluorescence. The second step reagent was a fluoresceinated goat anti-rabbit immunoglobulin (Tago, Inc.). PBMC were stained and analyzed with a fluorescent-activated cell sorter (FACS III, Becton-Dickinson, Sunnyvale, CA) as previously described. Goat anti-human IgG (Fab)<sub>2</sub> was used to quantitate B cells. Monocyte proportions were determined by fixing air-dried smears of PBMC and staining for nonspecific esterase (22).

**Typing of HSV isolates with monoclonal antibodies.** HSV isolates from patients' lesions were cultured in Hep-2 cell monolayers in cluster well plates for 24 h. The cells were removed from cluster wells by scraping, applied to slides fixed in acetone for 5 min and stored at -70°C for later staining. After refixation in acetone, the slides were incubated in a humidified chamber at room temperature for 30 min with anti-gA/B monoclonal antibody, which cross-reacts with both types I and II HSV, and anti-gD (type specific [ts]) and anti-gC antibodies (II-73) specific for type I HSV (18, 19). The monoclonal antibody to gD used here (II-436) was type specific (for HSV I) and hence completely different from the type common antibody (III-174) used to detect gD of HSV I or II in tissue sections. Use of two type-specific antibodies minimized typing errors caused by intratypic antigenic variation (23). Antibodies were diluted 1:15 and 1:50 in human serum seronegative for HSV to reduce nonspecific binding to Fc receptors. After washing twice with PBS, the slides were incubated for a further 30 min with fluoresceinated goat anti-mouse (Fab)<sub>2</sub> antibody, washed twice, and then mounted with 10% PBS in glycerol. Cells infected with standard laboratory F (type I) and G (type II) strains (obtained from Dr. B. Roizman) as well as wild type I and II HSV infected cells (typed by restriction endonuclease analysis in the laboratory of Dr. A. Arvin, Stanford University) were included as controls. Cells with fluorescent cytoplasm were detected with a Zeiss photomicroscope II (Carl Zeiss, Inc., Thornwood, NY) using epiillumination. The pattern typical for type I infected cells was anti-gA/B positive, anti-gD ts, and gC positive, whereas type II infected cells were anti-gA/B positive, anti-gC, and gD (ts) negative.

**Culture of PBMC for interferon.** PBMC separated by Ficoll-hypaque gradients were cultured in microtiter plates with or without HSV antigen for 2 d as previously described (4). Aspirated pooled supernatants were assayed by plaque reduction in human foreskin fibroblasts using vesicular stomatitis virus as the challenge virus. A laboratory reference standard leukocyte interferon (equivalent to 2,000 units/ml of the National Institutes of Health standard) was run with each assay.

Table I. Specificity of Monoclonal Antibodies Used in Immunoperoxidase Staining of Tissue Sections

Antibody	Dilution	Cell specificity	Source and references
Anti-Leu 2a	1:60	"Cytotoxic/suppressor" T cells	Becton-Dickinson (Mountain View, CA); 10
Anti-Leu 3a	1:60	"Helper/inducer" T cells	Becton-Dickinson; 10
Anti-Leu 4	1:30	All T lymphocytes	Becton-Dickinson; 11
Anti-Leu 6	1:50	Langerhans cells	Becton-Dickinson; 12
Anti-Leu 7	1:60	Most K and NK cells	Becton-Dickinson; 13
Anti-Leu 8	1:100	80% Leu 3 <sup>+</sup> cells 60% of Leu 2 <sup>+</sup> cells	Edgar Engleman, (Stanford, CA); 14
Anti-Leu M3	1:60	Monocytes/macrophages and Langerhans cells	Becton-Dickinson, 15
L203 (anti-HLA-DR)	Supernatant	Macrophages, B cells, activated T cells, endothelium	Ronald Levy (Stanford, CA); 16
T015	Supernatant	All B lymphocytes	David Mason (Oxford, United Kingdom); 17
Anti-gA/B	1:50	Monoclonal antibody to glycoprotein A/B of HSV (II-137)	Michael Para; 18, 19
Anti-gD (type specific)	1:50	Monoclonal antibody to glycoprotein D (type common epitope) of HSV (III-174)	Michael Para; 18, 19
Anti-alpha interferon	1:100	Polyclonal rabbit antisera	Sydney Pestka (Nutley, NJ)
Anti-gamma interferon	1:100	Polyclonal rabbit antisera	Jan Vilcek (New York)

## Results

**Clinical findings.** The nine patients presented from <12 h to 6 d after the first symptoms or signs of their recurrent herpetic lesions (Table II). Only two had prodromal symptoms. The age of the specific vesicle or crust biopsied was carefully determined. This was facilitated by the small number of sites involved in each lesion (8:9 patients had less than five vesicles or crusts). Patient 1 was examined first during a diffuse prodrome and had no visible lesion. An erythematous papule developed overnight and was biopsied next morning. The patients in this study had suffered recurrent peripheral herpes from 4 to 40 yr with recurrence rates varying from once every 2 yr to 8–10 per yr. The usual precipitants of stress and sun exposure were noted in six of the nine patients. The others could not identify precipitating factors. Three patients had a family history of recurrent herpes labialis or genitalis.

**HSV isolation and typing.** The site of the lesions, their condition at biopsy, and the HSV type isolated are noted in Table II. Three patients were infected with type I and six with type II HSV. All patients with type I and one with type II had recurrent lesions within 1 mo of biopsy. HSV type could not be predicted by anatomic location. Patient 6 had coexisting recurrent herpes labialis and right posterior thigh herpes since childhood. The isolate from the latter was type I.

**Phenotyping of PBMC.** No patient's PBMC spontaneously produced interferon gamma at either bleed. Blood "helper-suppressor" ratios (Leu 3<sup>+</sup> cells/Leu 2<sup>+</sup> cells) have been reported to be low (<1.0) in the first 4 d of a herpetic lesion (24). Although the mean ratio was lower at less than 2-d duration (1.4±0.5) as compared with later times (2.1±0.4), the correlation between ratios and time was not significant (Spearman rank correlation coefficient  $r_s = 0.55$ , NS). The lowest ratio was 1.0 in this small series.

**Histology (Table III).** Biopsies of early lesions or biopsies taken adjacent to a vesicle showed nuclear chromatin clumping and margination and cytoplasmic vacuolation. One late lesion

(patient 9) showed similar changes. In later lesions, there was epidermal cell necrosis and vesicle formation with multinucleated epidermal cells (Fig. 1) and occasional nuclear inclusions typical of herpes simplex infection. Cell debris and macrophages were present in advanced lesions occasionally associated with small numbers of neutrophils. Subepithelial diffuse mononuclear infiltrates were present in all sections although they varied in density (Fig. 1).

**Immunoperoxidase staining (Table III).** The mononuclear infiltrates consisted almost entirely of Leu 4<sup>+</sup> T cells with small numbers of Leu M3<sup>+</sup> monocytes (Fig. 2). Mean cell counts per patient biopsies ranged from 190 to 1,890 T cells and 30–140 monocytes. The majority of the cells were DR<sup>+</sup> (Fig. 2). Leu 3<sup>+</sup> cells were more numerous than Leu 2<sup>+</sup> cells in all sections. Mean cell counts ranged from 165 to 1,340 Leu 3<sup>+</sup> cells and 26 to 226 Leu 2<sup>+</sup> cells. Comparison of Leu 3<sup>+</sup>/Leu 2<sup>+</sup> T cell ratios in the lesions and blood of these patients showed much higher ratios in lesions biopsied at <2 d (Fig. 3). Thereafter, the ratios equalized. Overall, the tissue ratios were significantly more frequently greater than blood ratios ( $P < 0.05$ , Wilcoxon matched pairs signed ranks test, two-tailed) and there was a significant negative correlation between tissue ratios and age of lesion at biopsy (Spearman rank correlation coefficient with correction for tied ranks,  $r_s = -0.781$ ,  $P < 0.02$ , two-tailed). The proportion of Leu 8<sup>+</sup> cells was approximately equal to that of Leu 3<sup>+</sup> cells. Since Leu 8 recognizes 80% of Leu 3<sup>+</sup> cells, this suggested that there was no selective accumulation or depletion of these cells. Little variation in cell proportions was found in different areas of the sections particularly between subepidermal and deep focal perivascular infiltrates, except for Leu M3<sup>+</sup> monocytes/macrophages which were more numerous in subepidermal infiltrates deep to vesicles (two containing HSV antigen), ulcers, or crusts. Small members of Leu 3<sup>+</sup> and Leu 2<sup>+</sup> lymphocytes were observed within the basal layers of the epidermis. Percentages of Leu M3<sup>+</sup> cells in tissue mononuclear infiltrates and those of esterase-positive monocytes in PBMC were similar

Table II. Description of Lesions, Culture Isolates, and Immunotyping of PBMC

Patient No.	Days post onset	Site of herpes	Condition at biopsy	HSV type	Lymphocyte subset and monocyte proportions in blood
					Leu 4/Leu 2/Leu 3/slg <sup>+</sup> /Est <sup>+</sup> *
					%
1	12 h	Left upper chest	Erythematous papule	II	75/34/44/12/15
2	1	Right elbow	Erythema, new vesicles	II	55/19/35/12/13
3	1	Right elbow	Erythema, new vesicles	II	64/36/37/8/11
4	2	Large lesion on forehead	Erythema and vesicles	I	74/24/41/9/14
5	2	Right buttock (small)	Erythema, early vesicles	II	68/20/50/11/11
6	2	Right post thigh	Erythema, early vesicles	I	64/21/43/14/10
7	3	Right scapula	Erythema, crust	II	67/19/51/12/12
8	5	Anterior left shoulder	Erythema, crust	II	76/24/51/13/11
9	6	Right index finger, palmar aspect	Cloudy vesicle, erythema	I	68/26/41/14/10

\* slg<sup>+</sup>, surface immunoglobulin positive; Est<sup>+</sup>, nonspecific esterase positive.

Table III. Histology of Lesions, Immunophenotyping of Mononuclear Infiltrate, and Identification of HSV Antigen and DR<sup>+</sup> Epithelium

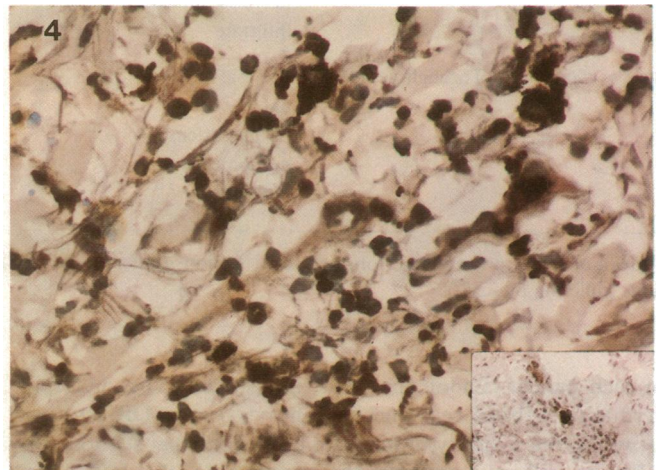
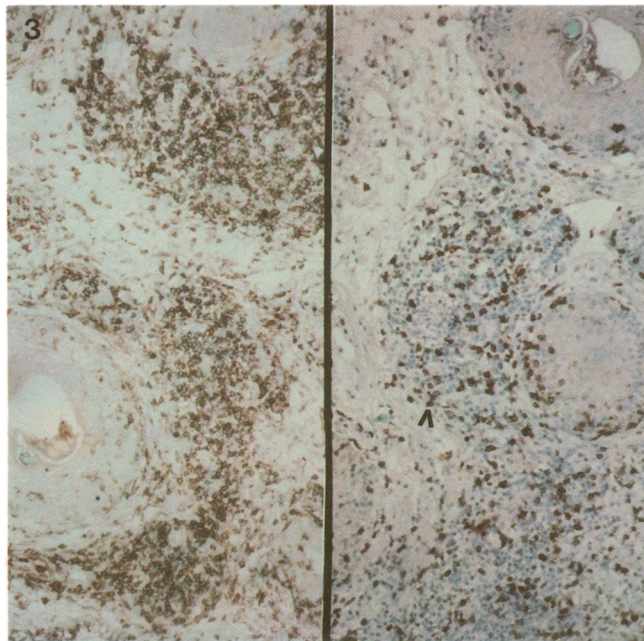
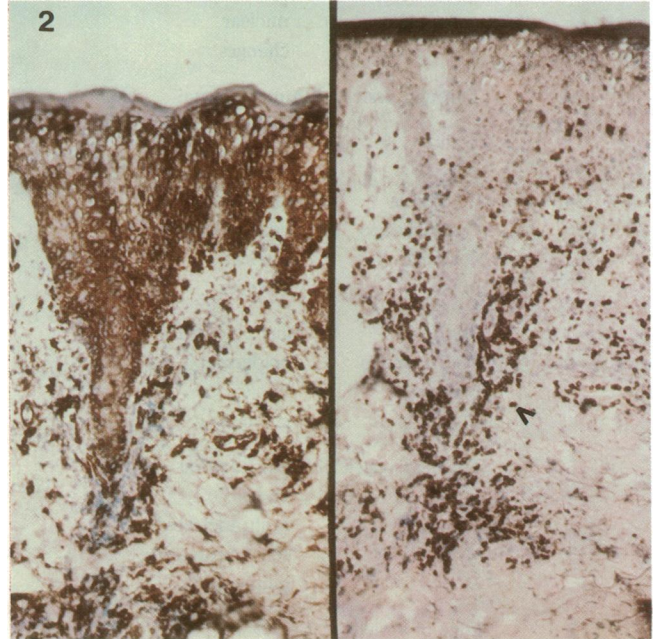
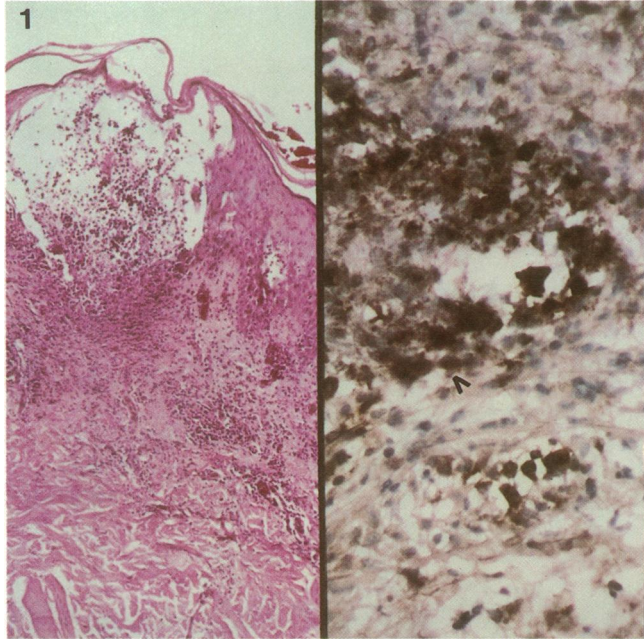
Patient No.	Time after lesion onset	Site of biopsy	Histology	Immunoperoxidase staining						
				Leu 3/Leu 2* (tissue)	Leu 3/Leu 2 (PBMC)	M3 (tissue)	Leu 7	Antibody to gA or gD	Antibody to interferon gamma	DR <sup>+</sup> epithelium
1	12 h	Through erythematous papule	Intact epidermis, epidermal nuclear changes	5.9	1.3	9	1	-	ND	Mostly -, weak focal +
2	24 h	Through vesicle	Intraepidermal vesicle, mixed infiltrate	6.3	1.9	12	1	+	+	Mostly -, focal +
3	24 h	Erythema adjacent to vesicle	Intact epidermis, epidermal nuclear changes	3.4	1.0	10	2	-	ND	-
4	2 d	Erythema adjacent to vesicle	Intact epidermis, epidermal nuclear changes	3.3	1.7	7.5	4	+	+	++
5	2 d	Through vesicle	Intraepidermal vesicle, multinucleated cells chromatin margination	2.4	2.5	14.5	2	+	++	Focal +, not diffuse
6	2 d	Edge of vesicle	Epidermal nuclear changes	2.5	2.0	13	1	-	++	++
7	3 d	Through ulcer/crust	Focal ulcer, mixed infiltrate	2.4	2.7	9	4	+	+	++
8	5 d	Through crust	Epidermal necrosis, mixed infiltrate	2.3	2.1	8	4	-	+	++
9	6 d	Through vesicle	Epidermal nuclear changes, dense mononuclear infiltrate	2.5	1.6	7	4	-	ND	++

\* Mean of Leu 3 proportions from two serial sections (differing by <5%) divided by mean of Leu 2 proportions from two adjacent serial sections (differing by <5%).

in each patient. Endothelial cells, Langerhans cells, and epidermal cells (basal cells and keratinocytes) were DR positive as well as the majority of infiltrate cells. Epithelial staining for DR was diffusely and strongly positive in the late stage biopsies. However, biopsies taken through vesicles at 12–24 h showed only weak focal staining of the epithelium for DR and the biopsy taken adjacent to a vesicle at 24 h showed no DR<sup>+</sup> epithelium. Comparison of DR and Leu 6 staining on sequential sections demonstrated diffuse staining of epithelial cells and intense staining of Langerhans cells which also were stained by Leu 6. Six biopsies of normal skin stained with the same

antibody (L203) concurrently showed no DR antigen expression. B cells (TO15<sup>+</sup>) and Leu 7<sup>+</sup> (NK/K) cells were rare in all sections.

In 6:6 biopsies stained with antibody to interferon gamma (or a substance copurifying with it), there were scattered positive large macrophages and small cells, probably lymphocytes (Fig. 4). Positive cells were more numerous in patients 5 and 6 and were occasionally seen within vessels (Fig. 4). Membrane staining of small cells was also observed in all six cases. No cells stained positively with the polyclonal antibody to human interferon alpha. Smears of unstimulated PBMC



did not stain with either antibody. Although HSV antigen-stimulated PBMC produced interferon alpha (unpublished observations), smears of these cells did not stain with the antibody to interferon alpha. Approximately 1 in 200 of the PHA-stimulated PBMC showed sparse cytoplasmic fluorescent granules when stained with the antibody to interferon gamma.

HSV glycoprotein antigen was detected in four patients' biopsies, usually within necrotic epidermal cells of the lower strata, and within vesicles. No antigen was detected in cells identifiable as Langerhans cells or macrophages.

## Discussion

In this study of peripheral recurrent herpes simplex lesions, we used immunoperoxidase staining with monoclonal antibodies to demonstrate the predominant mononuclear cell types within the dermal infiltrates, the change in their proportions with time, their ability to produce or take up interferon gamma, and their morphologic relationship to cells containing HSV antigen. The histology of recurrent herpes labialis at successive stages of the lesions has been reported recently by Huff et al. (6). In peripheral herpes simplex lesions caused by HSV I or II, we have found similar epidermal changes, commencing with changes in nuclear chromatin and progressing to focal necrosis of keratinocytes and intraepidermal vesicle formation. As in their study, we also found diffuse mononuclear subepidermal cells, but we also observed intradermal focal mononuclear infiltrates and few polymorphonuclear cells in the infiltrates only in the presence of extensive necrosis. Intradermal focal perivascular mononuclear infiltrates have not been previously reported in recurrent herpetic lesions. Our immunohistologic findings confirmed the presence of HSV glycoprotein antigen within the nuclei and cytoplasm of keratinocytes and basal epidermal cells. However, the antigen was mostly detected in necrotic epidermal cells and only in biopsies taken from vesicular lesions at 1–3 d. HSV concentrations are maximal in lesions at this stage (25). Despite the use of antibodies to two HSV glycoproteins, only high concentrations of intracellular antigen, late in the cell virus cycle, were detected. Immunologically important but lower concentrations of HSV antigen may be present in macrophages or Langerhans cells although not detected with this technique. Choice of biopsy site and variations in strain reactivity with the monoclonal antibodies used may also influence antigen detection.

Immunophenotyping of the subepidermal mononuclear infiltrates suggested T cells and monocytes migrated selectively into the lesions, as virtually no B cells and very low numbers of Leu 7<sup>+</sup> (NK/K) cells were detected. In contrast, PBMC of normal controls usually have 15±7% (mean±1 SD) (13) Leu 7<sup>+</sup> cells and 9±5% Ig + B cells (8–14% sIg<sup>+</sup>B cells in this study) (26). M3<sup>+</sup> monocytes or macrophages were present in similar proportions to those in blood. A slightly greater predominance of these cells was seen adjacent to vesicles, ulcers, or crusts. Their function may be phagocytosis and/or processing and presenting of HSV antigen derived from infected epidermal cells. The majority of the infiltrate cells at all stages were DR<sup>+</sup>. Allowing for most monocytes or macrophages being DR<sup>+</sup>, a large proportion of the T cells expressed DR indicating their activated state. Similarly it has been shown that most T cells in leprotic skin lesions are activated (7).

Further subdivision of T cells into their Leu 3<sup>+</sup> (helper) and Leu 2<sup>+</sup> (cytotoxic/suppressor) subsets revealed convergent progressive changes in the Leu 3<sup>+</sup>/Leu 2<sup>+</sup> ratios of tissue and PBMC. The initial disparity between tissue and blood ratios suggests an early selective migration of Leu 3<sup>+</sup> T lymphocytes into the lesion, producing the bulk of the early intradermal infiltrate. The later decrease in tissue ratios at 2 d after lesion onset might be explained by cessation of an early chemotactic stimulus to Leu 3<sup>+</sup> cell migration or development of a second attractive stimulus to cytotoxic or suppressor cells or both. Although proximity of mononuclear cells to HSV antigen-containing cells was observed (Fig. 1) and despite the fact that cytotoxic T lymphocytes recognizing the glycoprotein antigens in infected cells have been seen in animal HSV infections, no selective concentration of Leu 2<sup>+</sup> cells adjacent to vesicles was seen at any stage (27).

Antibody to interferon gamma (or a substance copurifying with it) stained the cytoplasm of macrophages and probably T lymphocytes within dermal infiltrates and occasionally vessels. Rim or membrane staining of lymphocytes was also observed. This staining pattern is similar to that observed for interleukin 2 in cytologic preparations (28) where producing cells showed cytoplasmic staining and adsorbing cells showed membrane staining. If the substance identified by staining was interferon gamma (or another lymphokine), cytoplasmic staining of small cells in the herpetic lesions probably also indicated synthesis but the cytoplasmic staining in macrophages could also have resulted from uptake. "Membrane" stained cells may be

*Figure 1.* Recurrent cutaneous herpes simplex: intraepidermal vesicle with multinucleated cells and dermal mononuclear infiltrate (H + E, × 120). Inset (right): Herpes simplex glycoprotein A/B antigen stained brown within nuclei and cytoplasm of necrotic epidermal cells (arrow) in the base of a vesicle. Note the peroxidase-negative inflammatory cells whose nuclei are counterstained blue with methylene blue in the underlying dermis. (Patient 5, immunoperoxidase, × 250).

*Figure 2.* (Left) DR antigen-positive epidermis and cells of subepidermal infiltrate. Langerhans cells are the most intensely staining cells in the epidermis. Note brown "ring" (cytoplasmic) staining of infiltrate cells. (Patient 6, immunoperoxidase, × 190). (Right) Leu 4<sup>+</sup> T lymphocytes in intradermal mononuclear infiltrates: 85–90% of cells are stained brown (arrow). (Immunoperoxidase, × 120).

*Figure 3.* (Left) Leu 3<sup>+</sup> (helper) T lymphocytes stained brown in intradermal mononuclear infiltrate adjacent to transected epidermis. (Patient 4, immunoperoxidase, × 190). (Right) Leu 2<sup>+</sup> (cytotoxic/suppressor) T lymphocytes (arrow) in intradermal infiltrate adjacent to hair follicle. The nuclei of the more numerous nonstaining cells are counterstained with methylene blue. Note Leu 2<sup>+</sup> cells (cells with brown "ring" staining) are less numerous than Leu 3<sup>+</sup> cells. (Patient 4, immunoperoxidase, × 400).

*Figure 4.* Large (macrophages) and small (probably T lymphocytes) cells in intradermal infiltrates with cytoplasmic staining for a substance copurifying with interferon gamma. (Immunoperoxidase, × 190). Inset: small cells, probably lymphocytes, exhibiting positive staining for this substance within a dermal vessel. (Immunoperoxidase, × 250).

adsorbing and responding to a lymphokine. In view of the minor contamination of the native interferon gamma immunogen with lymphotoxin and human IgG, these results must be confirmed with monoclonal or other antibodies to pure interferon gamma. However, in the absence of B cells, the cytoplasmic staining of small cells is unlikely to represent IgG or in early biopsies, lymphotoxin. The reasons for lack of staining of cells either in smears or tissue sections with the antibody to interferon alpha are unclear. Alpha interferons in high titer (up to 60,000 units/ml) have been consistently found in resolving herpetic vesicles and are probably produced by infected keratinocytes or adjacent mononuclear cells (29).

The presence of cells with cytoplasmic staining in dermal vessels as well as intradermal infiltrates suggests that these cells circulate and may be secreting interferon gamma. In previous studies of recurrent herpes labialis, we showed interferon gamma was produced spontaneously by circulating Leu 3<sup>+</sup> nylon wool-adherent T lymphocytes. The titer produced was directly proportional to time to next recurrence of herpes labialis (4). Although cells apparently synthesizing interferon were present within lesions and their vessels, we could not demonstrate spontaneous interferon production of >10 units/ml by PBMC in this study. However, six of nine HSV isolates were type II and two of the three patients with type I HSV recurred within 1 mo. Recurrence was usually associated with low interferon levels in herpes labialis. Differences in HSV antigen-specific T lymphocyte proliferation according to site of infection (lip or cornea) have been previously reported (1, 30). Hence, it is not clear whether HSV type, lesion site, or frequency of recurrence affects spontaneous interferon production. However, it is particularly interesting that the cell types required for HSV antigen induction of interferon gamma in vitro, macrophages, and Leu 3<sup>+</sup> T lymphocytes are those predominating in early herpetic lesion infiltrates.

The progressive increase in intensity and prevalence of epidermal cell DR expression was unexpected. Epidermal cells express DR antigen in certain skin diseases such as mycosis fungoides (31), lichen planus (32), and graft versus host disease (33) but it is not seen in normal skin, basal, or squamous cell carcinomas or alopecia areata (R. Turner and R. Warnke, personal observations). The only biopsy without DR<sup>+</sup> epidermal cells were taken early (24 h) and away from the vesicle edge. This finding and the progression of DR<sup>+</sup> staining suggests increasing local concentrations of a diffusible inducer. Purified interferon gamma increases DR antigen expression on both monocytes and melanoma cells which are of epidermal origin (34). This lymphokine also appears to be the major DR-inducing factor in concanavalin A-conditioned medium (35). Hence, after secretion by macrophages and T lymphocytes within the lesion infiltrates, it may induce DR antigen on the surfaces of epidermal cells. Interferon alpha also increases DR expression on monocytes in high concentrations in vitro and could contribute a local synergistic effect (36). Normal epidermal cells appear capable of presenting HSV antigen to T lymphocytes. This function was abolished by treatment with anti-DR antibody and complement. It was assumed that the DR antigen expressing Langerhan's cells were the antigen presenters (37). Perhaps, in recurrent herpetic lesions, DR antigen expressing HSV-infected keratinocytes or basal epidermal cells might present antigen directly to T lymphocytes. In support of this hypothesis, cells not of monocyte/macrophage

lineage but expressing DR antigen, such as Epstein-Barr virus-transformed B cells are also capable of presenting antigen (38). Interferon gamma, one of several lymphokines secreted by these cell types, may have important local immunoregulatory actions. It acts as an important macrophage-activating factor (39), increases D region antigen expression on monocytes (perhaps enhancing antigen presentation) (34), induces activation of cytotoxic T lymphocytes (40), and may induce expression of interleukin 2 receptors on T lymphocytes (41). The future availability of potent monoclonal antibodies to interferon gamma and other lymphokines and further functional subdivisions of lymphocytes should help elucidate the complex cellular and humoral events occurring in the recurrent herpetic lesion and the relevance of studies in animal models.

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