Lymphotoxin, Tumor Necrosis Factor, and Gamma Interferon Are Cytostatic for Normal Human Keratinocytes

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The effects of crude lymphokine-enriched supernatants, purified recombinant lymphotoxin (LT), tumor necrosis factor-α (TNF-α), and gamma interferon (γIF) on proliferating human keratinocytes were assessed using two in vitro culture systems. Activated splenocyte supernatants inhibited keratinocyte colony growth on fibroblast feeder layers and arrested basal keratinocyte DNA synthesis within 24 h. Purified recombinant LT, TNF-α, and γIF inhibited cell proliferation in serum-free medium without noticeably affecting viability. Cytostasis was dose-dependent (up to 90% with LT or TNF and 99% with γIF) and was maximal within 24–36 h. Specific antibodies neutralized TNF-α- and γIF-mediated cytostasis. Combined treatment with LT (or TNF) and γIF increased the degree of cytostasis, particularly at low lymphokine concentrations. Maximum inhibition of DNA synthesis and the duration of exposure required for this inhibition were comparable for LT and TNF and differed for γIF. Each of these lymphokines induced cell enlargement, flattening, and vesiculation, with γIF apparently more potent in this respect than LT or TNF. Fusiform keratinocytes with diffusely distributed cytokeratin were observed after prolonged treatment with γIF alone or γIF plus either LT or TNF. Flow cytometric studies of lymphokine-treated keratinocytes indicated that LT, TNF, and γIF could enhance β2 microglobulin expression 1.5-fold to threefold, whereas only γIF induced class II antigens. Staining for class II and β2 microglobulin was reduced on cells treated with high concentrations of γIF compared with either optimally treated or untreated cells. The potential relevance of these findings to cutaneous immune defense and disease is discussed. J Invest Dermatol 92:798–803, 1989

Immune cells produce a variety of hormonelike polypeptides (cytokines, lymphokines) that regulate hematopoietic cell activation, proliferation, and differentiation by complex interacting circuits. Because some lymphokines also affect epithelial and endothelial cells, they are thought to play key roles in expediting and localizing normal immune responses [1,2]. Increased understanding of the interactions between lymphokines and nonhematopoietic cells has focused attention on the idea that excessive levels or inappropriate combinations of lymphokines can produce inflammatory disease in otherwise healthy tissues.

Many dermatologic diseases are associated with lymphohistiocytic infiltrates whose pathogenic roles are suspected but poorly understood. Several studies suggest that lymphokines are present in epidermal lesions and that they may profoundly affect keratinocytes there [3–8]. This is especially true for gamma interferon (γIF), which is thought to occur, for example, in delayed type hypersensitivity (DTH), tuberculoid leprosy lesions, psoriasis, and cutaneous graft-versus-host disease (GVHD) [5–7,3,9]. The idea that γIF directly affects keratinocytes in vivo is consistent with reports that γIF inhibits growth of purified human keratinocytes and induces HLA-DR expression in vitro [10–13].

The direct effects of other immune cell cytokines on keratinocytes, especially those thought to be produced only by lymphoid and monocytic cells, are largely known. Two such cytokines are lymphotoxin (LT, TNF-β) and tumor necrosis factor (TNF, TNF-α, cachectin) [14–16]. Activated T cells secrete both factors, whereas macrophages secrete only TNF [16–18]. Lymphotoxin has long been considered a possible mediator of DTH reactions [19], and TNF has been linked to cutaneous acute GVHD [20]. Although LT and TNF are perhaps best known for their ability to lyse tumor cells and fibroblast cell lines, they also have distinct, cell type-specific effects on healthy cells [2]. Knowledge of keratinocyte responses to LT and TNF seems potentially important for the understanding of disease pathogenesis, especially as these lymphokines and γIF are co-produced by activated immune cells [17].

Here we report responses of normal human keratinocytes to in vitro treatment with crude and purified lymphokines. Splenocyte-conditioned medium and purified recombinant-derived LT, TNF, and γIF were tested for their effects on keratinocyte viability, proliferation, morphology, and expression of major histocompatibility complex (MHC) antigens. Each of these lymphokines inhibited

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keratinocyte proliferation in dose- and time-dependent fashion. Cytostasis was induced without overt cytotoxicity as indicated by analysis of cell recoveries, trypan dye exclusion, intracellular esterase, time-lapse microscopy, and lack of cell debris. In addition to their cytostatic effects, LT, TNF, and γIF induced conspicuous changes in keratinocyte cytology, MHC antigenicity, and adhesion. Compared with single lymphokine treatments, combined treatments with LT or TNF and γIF accelerated and intensified cytostasis and these cytologic changes.

**MATERIALS AND METHODS**

**Keratinocyte Culture** Epidermal sheets were removed from pieces of neonatal foreskin incubated overnight in 4 mg/ml Dispase (Boehringer, Mannheim, F.R.G.) at 4°C. Basal cells were released from sheets with trypsin (2.5 mg/ml in phosphate-buffered saline (PBS)), filtered through Nytex screen to remove aggregates, and washed into culture medium, and then plated onto plastic Petri dishes (Becton Dickinson Labware, Oxnard, CA) or multiwell flat-bottom plates (Corning, Corning, NY). All cultures were incubated at 37°C in humidified 7% CO₂, 93% air. Colony growth conditions were similar to those previously described [21]. The medium was Dulbecco’s modified minimal essential medium (Gibco Laboratories, Santa Clara, CA) supplemented with 20% fetal bovine serum, cholera toxin, epidermal growth factor, hydrocortisone, and 20% v/v of foreskin fibroblast-conditioned medium. Cells were plated onto dishes that had been preincubated and seeded with foreskin collagen [22]. Trypsinized, mouse 3T3 fibroblasts [21],respectively. Medium was replaced after 24 h and after every 2–7 d. Colonies were counted after 7–10 d after staining with rhodamine blue or according to Ayoub and Sklar [23]. Colony-forming efficiencies in culture controls ranged from 0.1–1.5%.

Alternatively, keratinocyte culture was performed using serum-free medium [24] with modifications [25]. Medium was constituted from powdered MCDB 153 medium base, vitamins, and trace element components (Irving Scientific, Santa Ana, CA). Additives were from Sigma (St. Louis, MO) except for O-phosphoethanolamine (Aldrich, Milwaukee, WI), bovine pituitary extract (U. of Colorado, Boulder, CO), and human recombinant epidermal growth factor prepared and generously provided by C. Georges-Nascimento (Chiron Corp., Emeryville, CA). Complete MCDB 153 contains these additives. Calcium concentrations were monitored by flame photometry. Keratinocytes were used at second or third passage (2–3 wk of culture). For proliferation assays, keratinocytes were seeded in complete MCDB 153 at 5 to 10 × 10³ cells/cm² into 96 or 24-well flat-bottomed plates or Petri dishes. One to 3 d later, the medium was washed with fresh medium containing added lymphokines or vehicle control. In some cases, lymphokines were preincubated with anti-TNF or anti-IFN antibodies (see below). Cells were incubated at the indicated times with 2.5 μg/ml of 3H-thymidine (3H-TdR) (2 Ci/m mole, Amersham International, Amersham, U.K.) for 16–24 h, trypsinized, and harvested onto glass fiber filters for scintillation counting or counted using a hemacytometer and phase-contrast microscopy.

**Lymphokines** Lymphokine-enriched medium (LEM) was obtained by culturing human splenocytes in 12 ng/ml of phorbol myristate acetate and 5 μg/ml of phytohemagglutinin for 90 min at 20°C, washing, and diluting to 3 × 10⁵ cells/ml for 40 h culture at 37°C in RPMI 1640 (Gibco) containing 3% pooled human serum, followed by 0.2 μm filtration of the culture supernatant. Recombinant human LT [26] was generously provided by Denka (Tokyo, Japan) through the kind efforts of Dr. Y. Kobayashi (National Cancer Institute, Frederick, MD). The LEM was supplied as a solution in PBS (lot 02-No1 containing 1.9 × 10⁶ LT units, 30 μg/ml, and 4 ng/ml of tritoxin) and was stored at 4°C. Recombinant human TNF-0.5 mg/ml, 2.5 × 10⁵ U/ml) and monoclonal anti-TNF antibody (lot 3314–16) were kindly provided by Genentech (South San Francisco, CA). Natural human γIF standard Gg23-901-530 was obtained from the NIAID Repository (Biotech Research Labs, Rockville, MD). Recombinant human γIF (batch 5-G1-42) was kindly provided by Dr. G. Seelig (Schering Corp., Bloomfield, NJ). It contained 100 to 200 endotoxin units per 3.5 × 10⁶ γIF units as supplied. Recombinant human γIF was also kindly provided by Shionogi Pharmaceutical Company (Osaka City, Japan). All γIF preparations were diluted in ice-cold 3% human serum albumin (Sigma) to 10³ to 10⁶ U/ml and stored at −70°C. Lymphokine-enriched medium was assayed for γIF using antibodies from hybridomas (B133.1, B133.3, and B133.5) generously donated by Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). A sandwich radioimmunoassay employing lysine/glutaraldehyde-coupled solid-phase antibody and natural γIF as standard was used. Cytotoxicity of LEM, LT, and TNF for actinomycin D-treated L929 fibroblasts was determined in 20-h assays using cells donated by Dr. N. Ruddle (Yale U., New Haven, CT). Crystal violet staining of surviving cells was measured in a dual-beam 96-well plate reader (Bio-Tek Instruments, Burlington, VT). Using this assay, the TNF was 10 to 50 times less potent than the LT per manufacturer’s stated unit. All purified lymphokine doses are presented here in terms of manufacturer’s units.

**Fluorescence Staining and Flow Cytometry** For cytokeratin staining, epidermal cells grown on glass slides were fixed 15 min in −20°C methanol and then incubated 15 min in ice-cold PBS containing 0.8% bovine serum albumin and 0.1% NaN₃ (PBS-BSA-N₃). Slides were incubated 45 min under 1/200 dilutions of hybridoma control or anti-cytokeratin ascitic fluids 34βB4, 34E4, or 35βH11 (gifts of Drs. A. Gown, University of Washington, Seattle, WA). Slides were washed briefly in PBS-BSA-N₃ before addition of a 1/50 dilution of fluorescein isothiocyanate-labeled goat antimouse Ig (TAGO Laboratories, Burlingame, CA), incubated 45 min, washed, and mounted under a coverslip with 90% glycerol in PBS. Observations were made using a Zeiss IM35 inverted epi-illumination fluorescence photomicroscope.

For flow cytometry, mouse hybridoma antibodies P4.1 (IgG₂a anti-DR) and 9.6 (control IgG₂a) were supplied by Dr. P. Martin (Fred Hutchinson Cancer Research Center). Hybridomas producing antibodies BBM.1 (IgG₂a anti-human beta-2 microglobulin), 10-3.6-27, and CRL8026 (both IgG₂a controls) were obtained from the American Type Culture Collection (Rockville, MD) and Dr. L. Herzenberg (Stanford U., Stanford, CA), respectively. Antibodies were purified from ascitic fluids according to Byet et al [27]. Trypsinated cells (2 × 10⁶) were stained indirectly by sequential, 1 h reactions with 100 μl of 5 μg/ml primary antibody followed by fluorescein isothiocyanate coupled goat antimouse IgG diluted 1/50 (TAGO) in diluent (see below) containing 0.5 μg/ml propidium iodide. Reactions were performed on ice in 96-well V-bottomed plates with intermittent agitation. The PBS-BSA-N₃ containing 2 mM EDTA was used as diluent and wash solution. Cells were analyzed on fluorescence-activated cell sorters (FACS II or IV; Becton-Dickinson, Sunnyvale, CA) equipped with a five decade logarithmic amplifier for fluorescence and right angle signals and a linear amplifier for forward angle signals. Data from 2 to 3 × 10⁴ cells were collected for each sample and subsequently computer processed to exclude data from dead cells (propidium iodide-stained) and cell aggregates.

**RESULTS**

**Supernatant of Activated Splenocytes Inhibits Keratinocyte Colony Formation** Activated immune cells secrete multiple lymphokine species. To learn how these might affect epidermal growth or viability, we assayed for its effects on keratinocyte colony growth on fibroblast feeder layers. The LEM was prepared from the supernatants of human splenocytes pulsed with phorbol myristate acetate/phytohemagglutinin. Direct cytotoxicity of LEM was tested by adding it to keratinocyte cultures 1, 3, or 5 d after plating (Table I). The LEM was not toxic for established (day 5) colonies, but it prevented colony formation early in culture. In addition, colonies grown in the presence of LEM were smaller and had more irregular borders compared with control colonies. Because its influence on established colony growth seemed to be
Table I. Keratinocyte Colony Inhibition After Delayed LEM Addition

<table>
<thead>
<tr>
<th>Day of Addition</th>
<th>% LEM</th>
<th>Colonies/Dish</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0</td>
<td>370 ± 12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>318 ± 12</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>74 ± 11</td>
<td>80</td>
</tr>
<tr>
<td>Day 3</td>
<td>0</td>
<td>443 ± 39</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>382 ± 9</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>199 ± 28</td>
<td>55</td>
</tr>
<tr>
<td>Day 5</td>
<td>0</td>
<td>429 ± 52</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>466 ± 17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>408 ± 66</td>
<td>5</td>
</tr>
</tbody>
</table>

Primary foreskin basal keratinocytes were plated at 5 × 10^5/cm^2 onto irradiated 3T3 fibroblast feeder layers. Cultures were fed with media containing the indicated concentrations of LEM on days 1, 3, or 5, and colonies were counted on day 8. Results are expressed as mean colonies ± SEM for triplicate cultures.

Table II. Specific Antibody Neutralizes TNF- and γIF-Mediated Cytostasis

<table>
<thead>
<tr>
<th>Lymphokine in Culture</th>
<th>Final Concentration (U/ml)</th>
<th>Lymphokine Preincubated With*</th>
<th>3H-Tdr Uptakeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>anti-TNF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>anti-γIF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>1 TNF</td>
<td>6300</td>
<td>+</td>
<td>2,699 ± 496</td>
</tr>
<tr>
<td>2 TNF</td>
<td>6300</td>
<td>+</td>
<td>13,159 ± 3,704</td>
</tr>
<tr>
<td>3 TNF</td>
<td>6300</td>
<td>+</td>
<td>2,341 ± 621</td>
</tr>
<tr>
<td>4 γIF</td>
<td>1000</td>
<td>+</td>
<td>11,818 ± 3,989</td>
</tr>
<tr>
<td>5 γIF</td>
<td>1000</td>
<td>+</td>
<td>199 ± 45</td>
</tr>
<tr>
<td>6 γIF</td>
<td>1000</td>
<td>+</td>
<td>180 ± 39</td>
</tr>
<tr>
<td>7 None</td>
<td></td>
<td></td>
<td>12,339 ± 2,824</td>
</tr>
</tbody>
</table>

* Equal volumes of lymphokines and antibodies (5 µg/ml) were incubated 2 h at 20°C before transferring 100 µl of the mixtures to adherent, 100-µl basal keratinocyte cultures.

b Results expressed as mean cpm ± SD for triplicate cultures pulsed with 3H-Tdr for 14 h beginning 26 h after lymphokine addition. Values reflecting lymphokine neutralization are underlined.

mainly cytostatic, the effect of LEM on keratinocyte DNA synthesis was tested in 3H-TdR incorporation assays. Keratinocytes were grown in serum-free MCDB 153 medium [24] for these and subsequent experiments. This medium was chosen 1) to provide proliferating basal keratinocytes, 2) to exclude effects of xenogeneic cells, sera, and other nonphysiologic culture components (e.g., cholera toxin), and 3) to avoid thymidine incorporation artifacts reported for keratinocytes grown in serum-supplemented medium [28]. Keratinocytes proliferated in MCDB 153 with a 24-h doubling time as judged by mitoses and cell counts. The LEM inhibited 3H-TdR uptake by these cells in a dose-dependent fashion. For example, treatment with 25%, 8%, or 3% LEM inhibited 3H-TdR uptake by 98%, 76%, and 49%, respectively. Cells were flattened and nonmitotic relative to controls after 24 h of exposure to 25% LEM.

Recombinant Lymphotoxin, Tumor Necrosis Factor, and Gamma Interferon Are Independently Cytostatic for Normal Basal Keratinocytes The LEM contained γIF (500 U/ml) and L929-cytolytic activity (at least 25 U/ml) (see Materials and Methods). Because anti-γIF and anti-TNF antibodies could partially neutralize the cytostatic activity of LEM, purified recombinant-derived lymphokines were individually tested for their effects on keratinocyte DNA synthesis and cell proliferation. Figure 1 depicts typical results obtained using recombinant LT, TNF, and γIF (left, middle, and right panels, respectively). Results from cultures labeled with 3H-TdR for 16 h starting on day 0 or day 1 are represented by the light and dark bars, respectively. Each lymphokine inhibited keratinocyte 3H-TdR uptake within 24 to 40 h (dark bars). The effect of γIF on 3H-TdR uptake differed somewhat from that of LT and TNF, in that γIF inhibited more than 99% of 3H-TdR incorporation, whereas maximum plateau inhibition by tested TNF and LT doses did not exceed 85–95% by day 2. Growth inhibition was dose-dependent and increased with time for all three lymphokines (compare light and dark bars). The TNF was more potent than LT when normalized to L929 cytolytic units.

To demonstrate that lymphokines per se were responsible for these results, cycling keratinocytes were exposed to lymphokines that had been preincubated with anti-TNF or anti-γIF monoclonal antibody, or medium alone (Table II). Cultures were harvested on day 2 after a 16-h 3H-TdR pulse. Anti-TNF neutralized cytostatic activity in the TNF preparation (compare lines 2 and 3), but did not affect γIF-mediated cytostasis (compare lines 5 and 6). Conversely, anti-γIF neutralized the γIF preparation (compare lines 4 and 6), but did not affect TNF-mediated cytostasis (compare lines 1 and 3). In all cases, proliferation in the presence of specifically neutralized lymphokines was restored to control levels (line 7). Specific antibodies were not available for analogous studies with LT.

Whether keratinocyte 3H-TdR incorporation accurately reflects cell proliferation has been questioned [28]. Therefore, cell counts were performed to confirm that these lymphokines were indeed cytostatic (Fig 2). The stippled bars represent cultures treated for 4 d (culture days 1 through 5) with the indicated γIF doses. Light bars represent results from parallel cultures that were not treated with lymphokine until day 3. The results confirmed that γIF treatment arrested or impeded keratinocyte proliferation. They also paralleled colony inhibition results obtained by delayed addition of LEM (see

Figure 1. Recombinant LT, TNF, and γIF independently inhibit human keratinocyte DNA synthesis. Proliferating foreskin basal keratinocytes were treated with the indicated doses of lymphokines. DNA synthesis was assessed at 24 h (light bars) and at 48 h (dark bars) after a 16-h pulse with 3H-TdR. Results expressed as mean cpm ± SEM for sextuplicate cultures. The largest error bars are shown.
Table 1). For example, cultures treated for 4 d with high-dose γIF (400 U/ml) showed no increase in cell numbers above initial input. The dependence of final cell numbers on γIF dose is consistent with the incomplete inhibition of ^3H-TdR incorporation noted above using lower γIF doses. In other experiments, cell recoveries 4 d after treatment of established cultures (4 × 10^6 keratinocytes/culture) with LT (50 U/ml), TNF (500 U/ml), γIF (5 U/ml), or medium only were 2 × 10^5 ± 3 × 10^5, 7 × 10^4 ± 8 × 10^4, 9 × 10^4 ± 4 × 10^4, and 3 × 10^5 ± 5 × 10^4 cells, respectively (mean ± SD for triplicate 10 cm^2 cultures; viability >90%). Toxicity of LT, TNF, or γIF for normal or actinomycin D-treated keratinocytes was not detected by cell counts or microscopy during 48-h cultures (Fig 2, see Discussion). However, toxicity for a minor subpopulation of cells at higher lymphokine concentrations was not excluded.

Combined Treatment with Lymphotoxin (or Tumor Necrosis Factor) and Gamma Interferon Enhances Keratinocyte Cytostasis Functional interactions between LT, TNF, and γIF were examined in studies using keratinocytes cultured for 2 d in media containing varied doses of these lymphokines. Typical examples of the interaction between LT and γIF are found in Figure 3. Here, it can be seen that combined treatment with LT and γIF was more inhibitory than treatment with LT or γIF at equivalent concentrations. Positive interactions between LT and γIF were observed over a broad range of concentrations (Fig 3). Analogous results were also obtained using mixtures of TNF and γIF as described below (Fig 4). Whether the positive interaction between LT (or TNF) and γIF was additive or synergistic depended on tested doses of each agent (i.e., synergy occurred at low lymphokine concentrations). The effects of LT and TNF were approximately additive when effective concentrations were normalized. Combined high-dose treatment with TNF and LT still allowed residual ^3H-TdR uptake similar in magnitude to that observed in the presence of high concentrations of either factor alone.

Additional experiments determined the duration of lymphokine exposure required for inhibition of DNA synthesis (Fig 4). Adherent keratinocytes were exposed to γIF or TNF, or both, for progressive intervals of time up to 8 h, and then washed into lymphokine-free medium (except for continuously exposed cultures). Cultures were pulse-labeled with ^3H-TdR during the last 16 h of a 40-h incubation period. Gamma interferon-treated cells showed a stronger correlation between the extent of cytostasis and exposure time than did TNF-treated cells. This suggests either that the proportion of susceptible target cells or the speed of action differs for these two lymphokines. The extent of growth arrest by γIF corresponded to the fraction of the keratinocyte cycle time (24 h) that
elapsed during exposure, which suggests that susceptibility to γIF-induced cytostasis is cell-cycle dependent. These results also demonstrate that combined treatment with TNF and γIF induced cytostasis more rapidly than either TNF or γIF alone. In a similar experiment, respective cell recoveries at 5 d after lymphokine treatment for 0, 0.5, 2, 4, 8, or 120 h were 16−, 13−, 13−, 11−, 10−, and 4-fold greater than cell input for TNF (2500 U/ml) and 18−, 6−, 4−, 2−, and 2-fold greater for γIF (50 U/ml). The effects of LT were similar to those of TNF in three parallel timed exposure experiments (data not shown).

Properties of Keratinocytes Treated with Lymphotoxin, Tumor Necrosis Factor, and/or Gamma Interferon Keratinocyte morphology was greatly altered after treatment for 2 to 3 d with LT, TNF, and/or γIF (Fig 5a−f). Lymphotoxin and TNF treatment increased total cell area and cell thickness over the nucleus. These two lymphokines also increased phase-contrast density and irregular cell borders. Cells treated with LT or TNF contained intracellular granules or vesicles, or both, approximately 1−2 μm in diameter (panel b). Gamma interferon treatment for 2 to 3 d induced extensive enlargement, cell flattening, and the development of large (> 5 μm), phase-refractile perinuclear vesicles in many cells. These cells initially resisted detachment by trypsin. Many cells remaining after prolonged gamma interferon treatment (> 3 d) were elongated (> 50 μm), fusiform, and attached to substrate only at each end (panel e). Combined treatment with γIF and either TNF or LT for 2 to 3 d (panel f) led to exaggerated changes in cell shape and vescularity resembling those observed after prolonged γIF treatment. Comparison of panels b and d with e (i.e., cells treated with TNF, low dose γIF, or both) again illustrates positive interaction between TNF and γIF. Treated cells reacted specifically with monoclonal antibody against squamous epithelial cytokeratin (panel f), but their diffuse cytokeratin organization differed from the normal fibrillar pattern of untreated cells. After 3 d, cell numbers gradually declined in lymphokine-treated cultures, but adherent cells remained viable at these concentrations of lymphokines according to the results of dye exclusion and intracellular esterase tests.

Induction of Keratinocyte Major Histocompatibility Complex Antigens Recombinant LT, TNF, and γIF were tested for their effects on keratinocyte cell surface HLA-DR and beta-2 microglobulin (β2-M). Beta-2 microglobulin (β2-M) was assayed to avoid overlooking potential changes in expression of non-HLA-A,B,C class I antigens [29]. Each column of flow cytometer contour plots in Figure 6 represents a different lymphokine treatment, and each row a different primary antibody for fluorescence analysis. Fluorescence intensity and forward light scatter are scaled along abscissas and ordinates, respectively. Effects of TNF were similar to those of LT in three consecutive experiments and are not shown. Treatment with LT or γIF caused cell enlargement, as indicated by displacement along the γ axis (Fig 6). Cell size heterogeneity in both the low-dose γIF and the LT-treated samples is indicated by the bimodal forward scatter values. More of the population was large (i.e., had greater scatter values) after γIF than after LT treatment. The fact that right angle scatter values were also greater for treated cells is consistent with the above microscopic observations of increased numbers of intracellular vesicles and granules in these cells. Lymphotoxin failed to induce class II (DR antigens) or to block class II induction by γIF (Fig 6, first row). Optimal γIF concentrations for 48-h class II induction were 10-fold higher than those required for 96-h expression. This suggests that dose and exposure time relationships for class II induction are interrelated. Maximum class II expression was obtained with γIF doses ranging from 4−100 U/ml, depending on the treatment day analyzed. Concentrations of γIF greater than 100 U/ml reduced the detected increase in class II antigen expression.

Cell surface β2-M was influenced both by the type and concentration of lymphokines employed (Fig 6, second row). Due to heterogeneous cell size and background staining in treated cell populations, specific staining was assessed relative to control Ig staining of the same-sized cell population in the same treatment group before comparison with similarly analyzed untreated keratinocytes. Using this procedure, LT treatment (600 U/ml) increased keratinocyte β2-M expression 1.5-fold compared with untreated cells. Low-dose γIF treatment (5 U/ml) increased staining of smaller cells for β2-M threefold, whereas staining of large cells in this same group increased only 1.25-fold. High-dose γIF treatment (500 U/ml) led to a threefold decrease in specific β2-M staining of the uniformly large cells. Thus, γIF appeared to induce keratinocyte membrane class I and class II antigens and then to cause cell enlargement with little or no additional MHC expression.

Figure 5. Recombinant TNF and γIF induce alterations in keratinocyte morphology. Forehead keratinocytes were exposed for 72 h to lymphokines in serum-free medium: a: no lymphokines; b: TNF (500 U/ml); c: γIF (1000 U/ml); d: γIF (10 U/ml); e: TNF plus γIF (500 U/ml plus 10 U/ml, respectively); f: similar to (e) but stained by indirect immunofluorescence with monoclonal 34BE12 antibody specific for squamous epithelial cell keratin (no staining was observed using isotype-matched control antibodies; see Materials and Methods). (bar: 50 μm for all panels)
DISCUSSION

This report demonstrates that LT, TNF, and γIF inhibit human basal keratinocyte proliferation in vitro. Low doses of γIF and either LT or TNF interacted to enhance cytostasis, and γIF alone acted more rapidly than LT or TNF. Although widespread toxicity of these lymphokines for keratinocytes was not observed, toxicity for a small subpopulation of cells cannot be excluded. For example, acute toxicity for cells in G2 or M would reduce total keratinocyte number by only 15% [30]. Time-lapse video microscopic studies, however, provided no evidence of acute toxicity for mitotic (shortly after treatment) or other keratinocyte subpopulations during 3 or 3 of treatment with these molecules. Furthermore, microscopic evidence of cell death (debris, phase-contrast, trypan blue uptake) was rare. Finally, direct cell counts showed that at least as many cells were recovered after 2 or 3 of treatment as were present before lymphokine addition. Given that such treatments blocked DNA synthesis, widespread cell death could not have occurred.

The mechanisms by which LT, TNF, and γIF inhibit growth of various transformed or virus-infected cells are unknown. It is unclear whether the LT- and γIF-induced accumulation of cells in G0/G1 reported for tumor cells [31] also occurs with keratinocytes. Growth inhibition of differentiating keratinocytes by γIF, which was previously reported [10,11], probably does not involve loss of the epidermal growth factor receptor, as expression of this receptor is increased on growth-inhibited, γIF-treated A431 cells [32]. Because basal keratinocytes can be uniquely affected by disease [33], we tested LT, TNF, γIF on cultures of proliferating basal cells. Each lymphokine was cytostatic, and keratinocytes seemed particularly sensitive to the effects of γIF, inasmuch as half-maximal inhibition of DNA synthesis occurred after treatment with 2 to 5 U/ml of recombinant γIF (Figs 1–4, Table II).

Lymphotixin and TNF synergize with γIF or enhance its effects in several systems [31,34] (Figs 3, 4). This is unlikely to reflect γIF-mediated LT/TNF receptor upregulation [35], as receptor number does not correlate with TNF sensitivity [36]. Synergy more likely involves complementary pathways affected by γIF and LT/TNF [31]. Synergy between TNF and interleukin 1 [37] in mediating cytostasis could also be involved in view of keratinocyte interleukin 1 production [38,39]. The relatively rapid cytostatic effects of γIF or γIF plus TNF/LT on keratinocytes (Fig 4) suggests that antilymphokine prophylaxis could be required to prevent epidermal damage by these lymphokines.

In addition to their cytostatic effects, LT, TNF, and γIF induced marked alterations in cell size, internal structure, substrate adherence, and cell surface MHC antigen expression. Changes in keratinocyte size and morphology are associated with dermal or epidermal lymphoid infiltration in vivo [5,3,9]. Cell flattening and spreading after in vitro γIF treatment may parallel epidermal thickening and keratinocyte enlargement that occurs during DTH [40], or after intradermal γIF injection [9]. Furthermore, the enlargement and vesiculation induced by lymphokine treatments (Fig 5) strongly resemble basal keratinocyte spongiosis and vacuolation observed in human acute GVHD [40]. Finally, changes in cell substrate attachment we observed after prolonged treatments with γIF or with γIF and LT/TNF may model in vivo events that precede epidermal sloughing.

Lymphokine-induced increases in MHC expression are of interest as possible stimuli to lymphocyte function in normal immunity and disease. DR antigen expression by γIF-treated cultured keratinocytes [12,13] suggests that keratinocyte class II expression in DTH and disease reflects in vivo γIF exposure. A role for LT or TNF in keratinocyte class II induction seems unlikely in view of the in vitro results reported here. Concerning MHC antigen induction by γIF, the present results differ from previous reports in two respects. First, HLA-DR was detected on virtually all γIF-treated keratinocytes (Fig 6), whereas others reported only 35 to 80% positivity [12,10,13]. Second, 10-fold lower concentrations of γIF (1–5 U/ml) than were previously described proved able to induce DR in this study. These discrepancies could be due to differences in media, keratinocyte differentiation states, or lymphokine stability or standards. Only subconfluent cultures were used in this study, and recombinant γIF preparations and National Institutes of Health standard natural γIF gave equivalent results. The fact that high-dose γIF treatment led to reduced relative increases of DR and actually decreased β2-M expression (Fig 6) may explain the reported failure of γIF to induce keratinocyte class I antigens [41]. Induction of class I after treatment with LT or TNF was less marked for keratinocytes than was previously reported for TNF-treated endothelial cells and fibroblasts [42].

Knowledge of keratinocyte lymphokine responses is important for understanding the role of epidermis in host defense and disease. Assuming that LT, TNF, and γIF also inhibit basal keratinocyte proliferation in vivo, they could normally act to arrest keratinocyte growth in virus-infected areas, limit virus production [43], and enhance MHC-restricted killing of infected cells. Although direct
evidence linking keratinocyte lymphokine responses to disease is lacking, such links seem likely to exist. One approach to this question hinges on the idea that lymphokines mediate cutaneous GVHD [44]. Strong parallels between basal keratinocyte cytopathology in GVHD and our in vitro findings (Fig 5) support this idea, as does the ability of anti-TNF to mitigate murine cutaneous GVHD [20]. The observations that rIFN and TNF have opposite effects on keratinocyte versus fibroblast proliferation in vitro (36, 45, this report), and that injected TNF preferentially localizes in mouse skin, liver, and gut [46], are intriguing in view of the fact that human GVHD is characterized by a sequence of epithelial atrophy and fibrosis in these organs. Evidence in support of this hypothesis is being sought in studies of keratinocyte cell-cycle phases and lymphokine levels in marrow transplant patients.

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