Activated Langerhans Cells release Tumor Necrosis Factor

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ABSTRACT

Langerhans cells act as antigen presenting cells in immune reactions in the skin. What other roles they may play in inflammation are less well defined. We have tested whether these cells can produce TNF-alpha, an important mediator of inflammation. Resting Langerhans cells produce less than 0.1 U TNF-alpha/ml. Langerhans cells stimulated with phorbol myristate acetate (PMA) and lipopolysaccharide (LPS) release 4-5 U TNF-alpha/ml. Specificity of the released TNF-alpha in an L929 cytotoxicity assay was confirmed using neutralizing anti-TNF-alpha monoclonal antibodies, and the identity of TNF-alpha was further confirmed by Northern blot hybridization using a TNF-alpha oligomer DNA probe. Activated Langerhans cells may contribute to inflammation in the skin by releasing TNF-alpha, which is known to effect fibroblast growth, endothelial cell activation, and lymphocyte function.
INTRODUCTION

Langerhans cells (LC) are mononuclear, dendritic cells located among suprabasal keratinocytes in the epidermis of all mammalian species. Although they represent only 2-6% of all epidermal cells they have a number of important roles in the initiation and maintenance of the immunoinflammatory response (1-2). For example they express class II antigens (3-5), the CD1 (OKT6) antigen (6) and the CD4 antigen (7) and they bear receptors for the Fc portion of IgG and several complement components (8). They appear to act as accessory cells in the induction of the mixed skin cell lymphocyte reaction (5,9).

Mononuclear phagocytes are known to produce and release a large number of biologically active cytokines (10). Two of these, interleukin 1 and tumor necrosis factor (TNF-alpha), are important mediators of the immunoinflammatory response (11). In the skin human keratinocytes are known to produce interleukin 1 alpha and beta, formerly called ETAF (epidermal cell-derived thymocyte activating factor) (12-13). LCs also release an IL-1 activity (14). We have tested whether these cells also produce TNF-alpha. Stimulated but not resting LCs contain mRNA for TNF-alpha and release this inflammatory mediator.
METHODS

Preparation of Dispersed Skin Cells

Single cell suspensions of normal skin were prepared from skin obtained at surgery as described previously (15-16). Briefly, trimmed skin was cut into 1 x 5 cm strips and split-cut with a Castroviejo keratotome set at 0.1 mm. The resulting slices were treated for 45 minutes at 37°C with 0.3% trypsin (M.A. Bioproducts, Walkersville, MD.) plus 0.1% EDTA in GNK (0.8% NaCl, 0.04% KCl, 0.1% glucose, 0.084% NaHCO₃, pH 7.3). Dispersed cells were suspended in Dulbecco's MEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS, 50 ug/ml gentamicin, and 2 mM L-glutamine (complete medium). Viability as determined by trypan blue exclusion immediately after trypsinization, was 80 percent or better.

Ficoll-Hypaque (F/H) Gradient Separation

Dispersed epidermal cells were resuspended at 4 x 10⁶ cells per ml in complete medium. Five ml of this cell suspension was layered on top of 3 ml Ficoll-Paque (Pharmacia, Piscataway, NJ) in a 15 ml tube. The tubes were centrifuged at 400 x g for 30 minutes at ambient temperature. The LC enriched interphase was collected, washed once, and resuspended in complete medium.
Panning

A 100 x 25 mm Lab Tek petri dish (Scientific Products) was coated with 10 ml of goat anti-mouse immunoglobulin (Zymed Labs Inc. South San Francisco, CA) at 10 μg/ml in 0.05 M Tris buffer, pH 9.5, for 40 min. hour and then washed three times with phosphate buffered saline (PBS) and once with 5% FCS/PBS. The epidermal cells were incubated with OKT6 monoclonal antibody (6) for 20 minutes, washed, resuspended in 5% FCS/PBS, layered on the coated petri dish surface, incubated at 4°C for 40 minutes, swirled, and incubated for another 30 minutes (16). The supernatant containing unattached cells was poured off gently, and the petri dish surface was washed five times with 1% FCS/PBS. The cells in the first three washes were pooled with the supernatant cells, and the cells in the last two washes were discarded. Attached Langerhans cells were scraped off with a rubber policeman in 1% FCS/PBS, centrifuged and resuspended in complete medium.

Induction of Natural Tumor Necrosis Factor (nTNF) from Langerhans Cells (LC).

Both LC enriched cells and LC depleted cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS). Phorbol myristate acid (PMA) was added at a final concentration of 100 ng/ml. The cells were incubated for 30 minutes at 37°C, collected by centrifugation, and resuspended in medium containing 10 μg of Escherichia coli 0111:B4 bacterial endotoxin (LPS). The cells were incubated at 37°C for 24 hours.
The culture supernatant were harvested and assayed in the TNF bioassay later the same day. Controls were the same cells without any stimulation.

**MTT Assay for Measuring Cytotoxic Activity.**

An MTT (1-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; M-2128 Sigma Chem. Co., St Louis, MO) cytotoxicity assay was used to measure inhibition of cell growth and % dead cells. In brief, murine L929 target cells were seeded in microplates, at a concentration of 4 X 10⁴ cells/well in 100 ul medium. Different dilutions of rTNF-alpha in RPMI-1640 was added to the target cells as standard control. The rTNF-alpha used in this study had a specific activity of 1 X 10⁷ U/mg protein in a 24 h bioassay with actinomycin D-treated L929 cells (17). After 18-20 h of incubation at 40°C, 20 ul MTT at a concentration of 2.5 mg/ml in phosphate-buffered saline was added and further incubated for 4 h at 37°C. After aspirating all the supernatant from the wells, 100 ul H₂O and 100 ul isopropanol with 0.04 N HCl was added to all wells. After dissolving the dark blue formazan crystals, the plates were read on a Dynatech microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Percentage of dead target cells was determined as:

\[
\text{% dead cells} = \frac{\text{optical density in wells with rTNF-alpha/supernatants}}{\text{optical density in control wells}}
\]
TNF-alpha Blocking Assay

A mouse anti-human TNF-alpha monoclonal antibody (15C8) was used in the blocking assay (18). Cytotoxicity assays were performed as described above and anti-TNF-alpha mAb was added into the well before adding supernatants to be tested. The control was the same isotype mouse anti-ricin monoclonal antibody (2D3). The percentage of cytotoxicity inhibition was calculated.

RNA Studies

RNA was prepared from 5 x 10⁶ cells using a single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-Chloroform extraction (19). RNA was run through Hybri-Slot™ blot on nitrocellulose membrane (BRL Cat. No. 1052MM) and hybridization was done with a kinased TNF-alpha (17) synthetic oligomer probe (sp. act. 2 x 10⁶ cpm/ug). The sequence used was: 5'--AAGGTCAACCTCCTCTCTGCTCCATCAAG--3'. Autoradiography was done using Kodak X-ray film XAR-5.

RESULTS

1. Stimulation of purified Langerhans cells releases cytotoxic activity.
Figure 1a presents a standard curve for the MTT-colorimetric microcytotoxicity assay using recombinant TNF-alpha. The sensitivity of this assay is 0.05 units of TNF-alpha. Figure 1b is a regression curve for the linear portion of the sigmoidal dose-response. The assay was linear over the range 0.025-12 units of TNF-alpha/ml. Table 1 shows that cultures enriched for Langerhans cells stimulated with PMA/LPS release 4-5 units/ml of TNF-alpha. Cultures depleted of LCs release much less TNF-alpha (0.1U/ml). Keratinocytes stimulated in the same manner as the LCs release even less TNF-alpha (0.03 U/ml). Cell lines were used for the positive (HL-60) and negative (K562) controls for these experiments.

2. Released cytotoxic activity is tumor necrosis factor.

To confirm that the released cytotoxic activity was TNF-alpha, monoclonal antibody neutralization studies were performed. Figure 2a shows the representative cytotoxic response of LC enriched and LC depleted cultures both stimulated and unstimulated with appropriate positive and negative control cell lines. Figure 2b demonstrates that the cytotoxicity released from stimulated Langerhans cells is neutralized by a specific anti-human TNF-alpha murine monoclonal antibody (18).

3. TNF-alpha mRNA is produced by stimulated Langerhans cells in vitro.

Figure 3 shows an mRNA slot blot of cells enriched for Langerhans cells stimulated with phorbol ester and lipopolysaccharide (lane 1-3). TNF-alpha mRNA is produced by
all the cells stimulated by PMA/LPS. Lane 1 shows the quantity of TNF-alpha mRNA from the original cell population isolated from fresh skin. Lane 2 is the TNF-alpha mRNA signal obtained from the Langerhans cell enriched population. The population of cells depleted of LCs shows a weaker signal (lane 3). The negative control K562 cells also produce some TNF-alpha mRNA after being stimulated in vitro (lane 4). The positive control HL-60 cells produce abundant TNF-alpha message (lane 5) Lanes 6-10 are the corresponding unstimulated cell populations. In all cases very little message for TNF-alpha is produced.

DISCUSSION

These studies demonstrate that purified Langerhans cells can be stimulated to produce and release TNF-alpha in vitro. The cytotoxicity of the released TNF-alpha can be neutralized by an anti-TNF-alpha antibody and LCs contain mRNA specifically hybridizing to a TNF-alpha oligomer probe. Unstimulated LCs and keratinocytes produce less than 0.1 U TNF-alpha/ml (below the detection sensitivity of the bioassay). A number of cell types including mast cells, mononuclear phagocytes and glial cells have been reported to release TNF-alpha (10). It is possible that the mRNA signal observed from the population of cells depleted of LCs is originating from keratinocytes. TNF-alpha has been recently shown to be a membrane-associated protein (20). Given the low quantity of TNF-alpha released by the LC depleted cultures it is possible that the relatively high levels of mRNA observed in the slot blot correspond to this form of the mediator. Further studies will be required to confirm this suspicion. It is also possible that the
population was incompletely depleted of LCs. However by immunoflorescent staining the depleted populations contained less than 1% LCs.

TNF-alpha is a pleiotropic mediator of the immunoinflammatory response (10). TNF-alpha is known to activate endothelial cells and granulocytes (21-22). Like IL-1, TNF-alpha promotes the growth of T cells and B cells when they have been stimulated with antigen (23-24) and may participate in antigen presentation (25). TNF-alpha (0.2-0.4 U/ml) is released during the mixed lymphocyte reaction (26), and as little as 10 U/ml augments thymidine incorporation by 60%. Thus local production of TNF-alpha by LCs has major consequences for the initiation and maintenance of the inflammatory response in the skin. Future studies will aim to determine the in situ localization of TNF in various diseases of the skin including psoriasis (27).

Acknowledgements

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REFERENCES


Table 1: Induction of TNF-alpha from Langerhans Enriched Populations of Cells

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Stimulation</th>
<th>+</th>
<th>-</th>
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<tbody>
<tr>
<td></td>
<td>(PMA/LPS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
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<tr>
<td>Langerhans enriched</td>
<td>4.53 ± 1.78&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>≤0.04</td>
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<tr>
<td>Langerhans depleted</td>
<td>0.10 ± 0.04</td>
<td>≤0.04</td>
<td></td>
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<tr>
<td>Keratinocytes</td>
<td>0.03 ± 0.01</td>
<td>≤0.04</td>
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<tr>
<td>Positive control (HL60)</td>
<td>2.65 ± 0.9</td>
<td>≤0.04</td>
<td></td>
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<tr>
<td>Negative control (K562)</td>
<td>≤0.04</td>
<td>≤0.04</td>
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<sup>a</sup> N=5 experiments

<sup>b</sup> TNF-alpha Units/ml; mean ± standard deviation
Figure 1:

a) L929 MTT cytotoxicity assay: rTNF-alpha standard curve.

b) Linear regression for the standard curve.

Figure 2:

a) Stimulated Langerhans cells release cytotoxic activity.

L929 assay demonstrating cytotoxic activity released from LPS and PMA stimulated or unstimulated control Langerhans cell populations. Populations enriched (+) or depleted (-) of Langerhans cell are shown. Cytotoxic activity of stimulated positive control HL60 and stimulated negative control K562 cells are also shown. Cytotoxicity above 20% is significant (see standard curve Figure 1). Representative of three experiments is shown.

b) Cytotoxic activity is neutralized by specific anti-TNF-alpha monoclonal antibody.

L929 assay demonstrating cytotoxic activity of LPS/PMA stimulated Langerhans enriched (+) and depleted (-) populations of cells is neutralized by specific anti-human TNF-alpha monoclonal antibody (15C8 at 40 ug/ml).
Figure 3:

Slot Blot Analysis of TNF-alpha mRNA Expression from Langerhans Cells.

RNA was prepared from $5 \times 10^6$ cells, slot blotted and probed with an end-labeled TNF-alpha specific oligomer probe as described in the methods.

Unseparated skin cell population-

Slot 1: LPS/PMA; slot 6: no induction

Langerhans enriched population-

Slot 2: LPS/PMA; slot 7: no induction

Langerhans depleted population-

Slot 3: LPS/PMA; slot 8: no induction

Negative control K562 cells-

Slot 4: LPS/PMA; slot 9: no induction

Positive control HL60 cells-

Slot 5: LPS/PMA; slot 10: no induction
rTNF Standard Curve

CYTOTOXICITY (%) vs rTNF (Unit/ml)
TNF CURVE FIT

\[ y = 100.70 - 8.8364x \quad R^2 = 0.982 \]

CYTOTOXICITY (%) vs. rTNF (Log Unit/ml)
nTNF

% CYTOTOXICITY

LC

ACTIVATION (PMA/LPS)

HL60 K562
NEUTRALIZATION BY ϵ TNF MAB

% CYTOTOXICITY

LC
ACTIVATION
MAB 15C8

15C8