Tumour necrosis factor- α enhances intraepithelial lymphocyte proliferation and migration

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Abstract

Background—Tumour necrosis factor α (TNF- α) is a proinflammatory cytokine found in abundance in diseased intestine.

Aims—The T cell production of TNF- α and the impact of this cytokine on intestinal T cell proliferation, migration, and cytotoxicity were studied.

Methods—Intestinal lymphocytes from normal jejunum were used. TNF- α production in culture supernates was measured by enzyme linked immunosorbent assay (ELISA). Lymphocyte proliferation was measured using ^3H thymidine uptake; migration, using transwell chambers; and cytotoxicity of HT-29 colon cancer cells, using the chromium-51 release assay.

Results—TNF- α was produced mainly by the CD8+ T cells in the intraepithelial lymphocytes (IEL) and the CD4+ T cells in the lamina propria lymphocytes in response to CD2 stimulation: 478 (94) and 782 (136) pg/ml, respectively. TNF- α (1 ng/ml or greater) augmented proliferation of IEL in response to interleukin 2 (IL-2), IL-7, or antibody to CD3 due to increased activation that did not involve IL-2 production or receptor generation. Conversely, antibody to TNF- α reduced IEL proliferation in response to IL-2 or IL-7. TNF- α also induced calcium mobilisation and chemokinesis (by 2.8 (0.5) fold over spontaneous migration). TNF- α had no effect on lymphokine activated killer cell activity.

Conclusions—TNF- α increases the proliferation and migration of IEL, which may expand their number in the epithelium.

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Keywords: CD8 positive T lymphocytes; tumour necrosis factor; lymphokine activated killer activity; chemokinesis, mucosal immunity; intestinal epithelium

Tumour necrosis factor α (TNF- α) has effects on many different cell types. It is a growth factor for T cells, B cells, and natural killer cells; it activates macrophages, neutrophils, and fibroblasts, stimulating release of interleukin 1 (IL-1), IL-6, and granulocyte/monocyte colony stimulating factor (GM-CSF). It promotes inflammation by triggering chemotaxis and upregulating adhesion molecules.¹⁻⁸ While most of its actions are proinflammatory, it can also impair T cell functions with chronic exposure.⁹

The intestine contains two major lymphocyte compartments: the intraepithelial lymphocytes (IEL) and the lamina propria lymphocytes (LPL).

TNF production in the mucosa has been studied most extensively in inflammatory bowel disease. Some studies found increased levels in diseased lamina propria compared with uninflamed bowel,¹⁰⁻¹² while another failed to detect any significant mRNA for TNF- α in these patients.¹³ TNF production by isolated cells is due to macrophages and T cells.^{12 14}

Treatment of chronic inflammatory diseases with antibody to TNF- α has some validity. In a rheumatoid arthritis model, for example, antibody given to mice with collagen induced arthritis improved the joint destruction and synovial hyperplasia. In patients with rheumatoid arthritis, antibody treatment improved symptoms and T cell mediated immune responses.^{15 16} Antibody to TNF- α also eliminated the intestinal pathology in murine salmonella infection and improved symptoms in Crohn's disease.^{17 18}

Since TNF- α has wide ranging effects on circulating lymphocytes and chronic inflammatory diseases, it is likely to be important in the intestinal immune response in health and disease. This study examines the production of TNF- α by intestinal lymphocytes and the ability of IEL to react to this cytokine.

Methods

ISOLATION OF IEL AND LPL

Intraepithelial lymphocytes were separated from normal appearing jejunal mucosa obtained from otherwise healthy individuals undergoing gastric bypass operations for morbid obesity. In brief, the minced mucosa was treated for 30 minutes at 37°C with 1 mM dithiothreitol (DTT) followed by three 45 minute incubations in a shaking water bath with 0.75 mM EDTA; the cells in the supernatants were collected. The IEL, purified by a Percoll density gradient, were found to be composed of over 90% lymphocytes (94 (5)% CD2+ and 89 (2)% CD8+).¹⁹

To isolate LPL, the treated tissue received three more 45 minute incubations with EDTA, and the released cells were discarded. The remaining tissue was digested for three hours at 37°C in 20 U/ml collagenase, then pressed through a wire mesh cell sieve to free the trapped cells. The LPL, purified using a Percoll density gradient, were 55 (10)% CD4+ and 35 (11)% CD8+.

Peripheral blood lymphocytes (PBL) were isolated from whole blood using Ficoll density gradient centrifugation. CD4+ and CD8+ T cell subsets were isolated by negative selection using immunomagnetic sorting.²⁰ The resulting populations contained less than 5% of the

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depleted cell type. For removal of CD4+ IEL, less than 1% contamination occurred.

PROLIFERATIVE STUDIES AND MEASUREMENT OF CYTOKINE PRODUCTION

Lymphocytes ($1 \times 10^5/0.1$ ml) were stimulated with phytohaemagglutinin (PHA, Burroughs-Wellcome, Greenville, North Carolina, USA), IL-2, IL-7, TNF- α (R&D Systems, Minneapolis, Minnesota, USA), phorbol myristate acetate (PMA), staphylococcal enterotoxin B (SEB), antibody to CD3 (Immunotech Inc., Westbrook, Maine, USA) and anti-T11₂ and anti-T11₃ antibodies (gift of E Reinherz, Dana-Farber Cancer Institute, Boston, Massachusetts, USA). Proliferation was determined after a three day culture by ³H thymidine uptake and expressed as a stimulation index (SI), the increase in cpm over that of an unstimulated control, which averaged 150 (70) cpm. Blocking experiments were performed by adding sheep antibody to TNF- α (5 μ g/ml, Sigma) at the initiation of the cultures.

Amounts of TNF- α and IL-2 were measured in culture supernates by enzyme linked immunosorbent assay (ELISA, Endogen Inc., Cambridge, Massachusetts, USA).

CALCIUM ION MOBILISATION STUDIES

Lymphocytes (5×10^6) were loaded with Fura-2 AM (Molecular Probes, Eugene, Oregon) for 20 minutes at 37°C, washed, resuspended to $1-2 \times 10^6$ /ml and placed on ice. After warming for five minutes, a 3 ml sample was transferred to a polystyrene cuvette. Fluorescence was monitored in a fluorescence spectrophotometer for one minute before and five minutes after the addition of each cytokine in a 30 μ l bolus using excitation wavelengths of 340 or 380 nm and monitoring an emission wavelength of 510 nm. Ionomycin (10^{-6} M) was added as a positive control. Cytoplasmic calcium ion concentration was calculated as the percentage of the maximum value with the dye fully chelated by the addition of ionomycin minus baseline values before the stimulus is introduced. In some experiments, the calcium specific chelator EGTA (2 nM) was added before the stimulus.²¹

MIGRATION ASSAY

The Boyden transwell system was used to measure lymphocyte migration in response to various factors. The upper and lower chambers were separated by a polycarbonate membrane free of polyvinylpyrrolidone (a wetting agent that retards cell adhesion), containing 5 μ m pores. Lymphocytes ($1 \times 10^5/0.05$ ml) were placed in the upper chambers and various concentrations of TNF- α were added to either chamber. After a four hour incubation at 37°C, the apparatus was kept at 4°C overnight to permit lymphocytes bound to the undersurface of the membrane to fall into the lower wells. It was then disassembled, and the numbers of lymphocytes in the lower chambers counted. The upper surface of the membrane was wiped free of lymphocytes, and the lower surface was documented to be free of cells by examination with light microscopy after fixing in 70% etha-

nol and staining with toluidine blue. Only viable lymphocytes (assessed by trypan blue exclusion) migrated through the membrane.²¹

LYMPHOKINE ACTIVATED KILLER ACTIVITY

Lymphokine activated killer (LAK) cells were generated by culturing IEL for three days with IL-2, with or without TNF- α or anti-TNF- α . The cells were then washed and incubated at various ratios with chromium-51 labelled HT-29 cells (American Type Culture Collection, Rockville, Maryland, USA). The percentage of cytotoxicity was calculated in relation to the spontaneous and maximal releases by target cells in medium and 2% cetrimide solution (Fisher), respectively, as detailed previously.²²

STATISTICAL ANALYSIS

For each set of data, a mean and SEM were calculated. Pairs of data sets were analysed by Student's *t* test for paired or independent variables.

Results

The amounts of TNF- α produced by IEL and LPL were measured by testing culture supernatants using a specific ELISA assay. Stimuli of the CD2 and CD3 receptors were used since intestinal lymphocytes show high and low proliferative responses, respectively, to these agents.¹⁹ Lymphocytes were cultured for 24 hours with the mitogenic antibodies to T11₂ and T11₃ (1/500 dilution) or antibody to CD3 (1 μ g/ml); the supernatants were collected after 24 hours and measured for TNF- α activity (fig 1). The amount of TNF- α produced was greater with LPL than IEL and greater with stimulation of CD2 than CD3. PBL produced 430 (120) pg/ml and 290 (93) pg/ml (*n*=3) with CD2 and CD3 stimulation, respectively.

The removal of CD4+ IEL, representing 5% of the whole, reduced anti-T11₂ and anti-T11₃ triggered TNF- α production by 33%, while removal of the CD8+ subset, representing 89% of the whole, reduced activity by 89%. Removal of CD4+ LPL, representing 55% of the whole, reduced cytokine production by 82%, while removal of the CD8+ T cell subset, representing 35% of the whole, only reduced production by 13%. Therefore, intestinal CD4+ T cells are more potent producers of TNF- α than are CD8+ T cells. Little TNF- α was produced by LPL stimulated with LPS (88 (23) pg/ml, *n*=3), suggesting that macrophages do not contribute substantially to TNF- α production.

IEL proliferation was then measured in response to several stimuli in the presence or absence of TNF- α (100 ng/ml) (fig 2). TNF- α increased IEL proliferation with IL-2, IL-7, and anti-CD3 antibody but not with PHA, SEB, PMA (SI of 75 (18) and 98 (17) with or without TNF- α , *n*=5), or the mitogenic antibodies directed against the CD2 molecule (SI of 91 (14) and 86 (9) with or without TNF- α , *n*=5). Even when the stimuli causing high mitogenic responses (PMA and CD2 ligation) were used at suboptimal doses, TNF- α was still unable to enhance the response (not shown), indicating that this effect

Figure 1 TNF- α , quantitated by ELISA, was measured on culture supernatants of IEL (A) or LPL (B) with medium, anti-TL α , and anti-TL β , antibodies (1/500 dilution), or anti-CD3 (1 μ g/ml) for one day ($n=3$).

was dependent on the type of stimulus rather than the degree of baseline cell division. Dose response curves indicated that the enhancing effect of TNF- α on IL-2 and IL-7 induced proliferation started at 1 ng/ml and reached a plateau at about 100 ng/ml (fig 3).

To determine whether the CD4+ or CD8+ T cell subsets of IEL responded to TNF- α , each subset was cultured with IL-2 and TNF- α and after a three day incubation, changes in proliferation were compared with control cultures in IL-2 alone. The increase in response by TNF- α on IL-2 induced cell division was equivalent for sham treated IEL and the CD8+ T cell subset (4.4 (0) fold and 4.4 (1.7) fold increases, respectively) but less for the CD4+ IEL (2.2 (0.4) fold, $p<0.05$, Student's t test, paired variables, compared with sham treated IEL, $n=3$). Since LPL contain a larger number of CD4+ than CD8+ T cells, they may be less responsive to TNF- α than are IEL. In fact, of the three stimuli that permit augmented TNF- α induced cell division by IEL (IL-2, IL-7, and antibody to CD3, fig 2), only IL-2 sensitised LPL to the TNF- α enhancement: 16 330 (2250) cpm with IL-2 versus 19 100 (2753) cpm with IL-2 and TNF- α ($p<0.05$, Student's t test, paired variables, $n=6$).

Another way of determining the effect of TNF- α on proliferation is to add blocking anti-

bodies to the assays (fig 4). Antibody to TNF- α reduced proliferation of IEL to IL-2 and IL-7, suggesting that endogenous TNF- α contributes to the cell division of IEL in response to these stimuli. However, this antibody had no effect on proliferation of IEL in response to PHA, SEB, or antibody to CD3.

To assess whether TNF- α enhances proliferation during IEL activation or cell division, the cytokine was added on days 0, 1, and 2 of a three day culture of IEL and IL-2 (fig 5). TNF- α had its optimal augmenting effect when added at the beginning of the culture. To determine whether TNF- α also affects activation events, IL-2 production and IL-2 receptor expression were measured in the presence or absence of this cytokine. After 24 hours, IL-2 production by IEL in response to IL-7 was minimal whether or not TNF- α was added: 20 (2) pg/ml with medium, 17 (4) pg/ml with IL-7, 19 (7) pg/ml with TNF- α , and 16 (7) pg/ml with IL-7 and TNF- α ($n=3$). Another activation event, IL-2 receptor expression triggered by IL-2 or IL-7, was also unaffected by TNF- α : the ratio of lymphocytes that developed IL-2 receptors with IL-2 and TNF- α compared with those with IL-2 alone was 1.06 (0.54) ($n=3$), which is not significantly different from 1.0. Therefore, TNF- α increases proliferation of IEL by affecting events in the first 24 hours independently of IL-2 production or receptor generation.

An additional measure of activation is the increased $[Ca^{2+}]_i$ in cells which occurs immediately on exposure to the stimulus (fig 6). Fresh IEL displayed an instantaneous rise in $[Ca^{2+}]_i$ followed by a sustained plateau. This response

Figure 2 IEL were cultured for three days with stimuli in the presence or absence of TNF- α (100 ng/ml). TNF augmented proliferation, measured by ^3H thymidine incorporation, only with certain stimuli. * $p<0.05$, $n=7$.

Figure 3 IL-2 induced proliferation of IEL was measured in the presence of various concentrations of TNF- α : at 1 ng/ml and above, TNF- α significantly increased proliferation. * $p<0.05$, $n=5$.

Figure 4 IEL were cultured for three days with stimuli in the presence or absence of antibody to TNF- α (5 μ g/ml). Proliferation was reduced with IL-2 and IL-7 as stimuli. * $p < 0.05$, $n = 6$.

was partially reduced by 40–60% with EGTA (which chelates extracellular calcium), indicating that the calcium influx is from both intracellular stores and extracellular fluid.

The migration of IEL towards TNF- α was measured after a four hour incubation in the Boyden chamber. When IEL (1×10^5) were placed in the upper wells and 100 ng/ml TNF- α was placed in the lower wells, the percentage of lymphocytes moving to the lower wells was 2.8 (0.5) fold greater than spontaneous migration towards medium alone ($1.1 (0.3) \times 10^5$ cells). To determine whether cell movement occurred only towards a higher concentration of chemokine (chemotaxis) or with exposure to chemokine regardless of the gradient (chemokinesis), IEL migration was measured with various concentrations of TNF- α in either upper or lower wells or both. With IEL placed in the upper chambers along with TNF- α , the respective migratory responses with medium or TNF- α in the lower chambers were 2.5 (0.4) and 2.7 (0.6) fold greater than spontaneous migration. The movement of IEL was the same whether

Figure 5 TNF- α (100 ng/ml) was added on days 0, 1, and 2 to IEL cultured with IL-2 or IL-7 ($n = 5$ and $n = 3$, respectively). Proliferation was increased only when TNF was added on day 0. * $p < 0.05$.

Figure 6 [Ca²⁺]_i fluxes in Fura-2 loaded IEL were measured by a fluorescence spectrophotometer using an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Changes induced by TNF- α (100 ng/ml) over a five minute period are denoted as a percentage maximum (fluorescence with dye fully chelated by ionomycin minus baseline values before the stimulus is introduced).

Figure 7 Cytotoxic activity of IEL cultured with IL-2 supplemented with either TNF- α (100 ng/ml) or antibody to TNF- α (5 μ g/ml) was measured at several effector to target (E:T) cell ratios against HT-29 colonic adenocarcinoma cells (representative of three experiments).

towards or away from the chemokine, establishing that chemokinesis was occurring.

The effect of TNF- α on LAK activity by IEL was then measured as TNF- α has been shown to induce cytotoxic activity (fig 7).^{23, 24} The cytotoxic activity against HT-29 colon cancer cells by IEL incubated for three days with medium, IL-2, or IL-7 was unaffected by TNF- α or antibody to TNF- α added at the initiation of the three day culture or during the assay (fig 7).

Discussion

TNF- α is produced mainly by T cells and macrophages. The present study shows that jejunal IEL and LPL produce larger quantities of TNF- α when stimulated through the CD2 rather than the CD3 pathway. IEL also secrete about 10-fold and 25-fold less TNF- α when stimulated by PHA or IL-2 than with CD2 ligation.²⁵ The CD4⁺ subset produced more cytokine than the CD8⁺ T cells, explaining why LPL secrete more than IEL. The discrepancy between TNF- α production triggered by CD2 versus CD3 stimulation has also been found using circulating T cells in one of two studies,^{14, 26} so may not be due to the heightened activation of intestinal lymphocytes through CD2. Culturing LPL with LPS, which stimulates macrophages and B cells, yielded a much lower amount of TNF- α than did T cell

stimuli. In inflammatory bowel disease, macrophages—defined by morphology, esterase positivity, and CD68 expression—are the main producers in the lamina propria.^{10–12} This cytokine, then, may be secreted mainly by T cells in the normal state and macrophages in inflammation. Secretion of TNF- α by IEL would increase epithelial permeability and cause epithelial cells to produce chemokines.^{27, 28} Production of TNF- α by LPL would recruit cells to the lamina propria and activate resident cells, such as mesenchymal cells and endothelial cells.^{4, 5}

This is the first study of the effects of TNF- α on IEL. This compartment of lymphocytes has substantially different features compared with circulating lymphocytes, such as their low proliferative response to CD3 ligation and to mitogens.¹⁸ TNF- α mainly affects intestinal CD8+ rather than CD4+ cells and IEL rather than LPL. This could be due to the lower proliferation and the lower production of TNF- α by the CD8+ cells and IEL. Alternatively, these cells may have more TNF- α receptors than the CD4+ cells and LPL. TNF- α at 1 ng/ml or greater enhanced proliferation of IEL in response to IL-2, IL-7, and anti-CD3 antibody. It did not affect proliferation in response to PHA, SEB, PMA, or mitogenic antibodies of the CD2 receptor. The last two induce high proliferative responses by IEL so further enhancement by TNF- α may not be recognised. However, stimulation of IEL with suboptimal concentrations of these agents still did not permit the TNF- α enhancement, suggesting that this effect is dependent on the type of stimulus rather than the degree of baseline proliferation. Alternatively, certain stimuli may trigger substantial TNF- α production so that addition of further TNF- α would be inconsequential. This could explain the lack of response by IEL triggered through the CD2 receptor which yields production of large amounts of TNF- α . When neutralising antibody to TNF- α is added to cultures, the resulting decline in cell division indicates that the stimulus induces TNF- α production which in turn triggers proliferation. This antibody reduced responsiveness of IEL to IL-2 and IL-7, but not to PHA, SEB, or antibody to CD3. This differential regulation may be due to the numbers of TNF receptors which are increased by IL-2 and decreased by T cell receptor activation.²⁹

Like IEL, the peripheral blood mononuclear cell (PBMC) response to IL-2 and anti-CD3 is augmented by TNF- α .⁷ Some studies found that TNF- α affected cell cycling, rather than activation events, since it increased proliferation when added 24 hours after initiating culture but did not alter IL-2 production. Other studies found that IL-2 receptor expression but not IL-2 production increased with TNF- α , suggesting that its effect is mainly on activation. With IEL, TNF- α enhances IL-2 and IL-7 induced proliferation within the first 24 hours during activation and induces an immediate calcium influx. It preserves IL-2 production and receptor generation. This cytokine, therefore, probably alters other nec-

essary activation events, such as stimulation of the transcription factor, nuclear factor κ B, and the interleukin receptor promoter.^{30, 31}

TNF- α induces chemokinesis by IEL and natural killer cells⁵ and chemotaxis by neutrophils and monocytes.⁴ Calcium influx is thought to be necessary for migration, and TNF- α induces both. However, other stimuli cause IEL to migrate in the absence of a calcium influx.²¹

TNF- α does not augment LAK activity by IEL against HT-29 cells, suggesting that it does not selectively cause proliferation of cytotoxic cells. However, TNF- α in combination with interferon γ causes apoptosis of these colon cancer cells.²³

The diverse biological actions of TNF- α contribute to inflammation but also to antibacterial activity and T cell mediated immunity. Its ability to effect tissue damage is shown by the tissue necrosis in T cell targeted TNF transgenes³² and by injection of TNF into intestinal segments.¹⁷ TNF- α , in contrast, aids in the development of antibacterial resistance and T cell mediated immunity.^{33, 34} These diverse effects are shown by TNF deficient mice which, when injected with *Corynebacterium parvum*, have little initial inflammatory response followed by inflammation that is delayed and prolonged.³⁵

Diseases of the jejunum, such as giardiasis and coeliac sprue, are characterised by increased numbers of lymphocytes (particularly IEL), villous atrophy, and crypt hyperplasia. Damaged epithelial cells may produce TNF which would increase IEL numbers by augmenting their proliferation and migration. TNF- α (perhaps in association with interferon γ) may destroy surface epithelial cells,³⁶ leading to shortening of the villi. TNF may also be mitogenic for crypt epithelial cells.³⁷ Many features of diseases of the jejunum may therefore depend on TNF- α .

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