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Triggered human mucosal T cells release tumour necrosis factor-alpha and interferon-gamma which kill human colonic epithelial cells

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SUMMARY

T cell activation can lead to local tissue injury in organ culture studies of human fetal jejunum, either directly through cytotoxicity or indirectly by the release of cytotoxic cytokines. The goal of this study was to establish *in vitro* whether cytotoxic cytokines can be released by isolated colonic T cells and what cytokine interactions are required for killing of human colonic epithelial cells. Cytokine-containing supernatants were induced by incubating unseparated lamina propria lymphocytes (LPL) or mucosal T cell subpopulations (separated by indirect panning) with anti-CD3 and/or K562 target cells for 18 h at 37°C. Cytokines were measured by cytotoxicity assays using L929 (murine fibroblast) and HT-29 (human colonic tumour) lines as target cells in combination with blocking anti-cytokine antibodies. Supernatants derived from unseparated, $CD4^+$ (>95% pure) and $CD8^+$ (>90% pure) LPL were cytotoxic to L929 targets (350 U/ml, 230 U/ml and 100 U/ml tumour necrosis factor-alpha, respectively). All or nearly all of the cytotoxicity was due to the presence of tumour necrosis factor-alpha (little or no tumour necrosis factor-beta was detected). These same supernatants were cytotoxic (up to 32% lysis at 1/4 dilution) to HT-29 targets in a 48-h ^{111}In release assay. Recombinant tumour necrosis factor-alpha and interferon-gamma alone produced minimal killing of HT-29, but together killed the HT-29 target cells. Anti-tumour necrosis factor-alpha or anti-interferon-gamma alone blocked killing of HT-29 target cells by LPL-derived supernatants, although anti-tumour necrosis factor-beta had no effect upon killing of HT-29. These results demonstrate that human LPL T cells, triggered by addition of anti-CD3 and target cells, produce tumour necrosis factor-alpha and interferon-gamma, both of which are required for optimal killing of HT-29. Simultaneous release of these cytokines in the vicinity of epithelial cells during immune responses could play an important role in the mucosal damage in chronic inflammatory states such as inflammatory bowel disease.

Keywords tumour necrosis factor-alpha interferon-gamma lamina propria lymphocytes T cell activation

INTRODUCTION

Lymphocytes in the lamina propria (LPL) differ from lymphocytes in the peripheral blood phenotypically (Shanahan *et al.*, 1988) and in their level of activation (Pallone *et al.*, 1987). As these cells are constantly exposed to foreign antigens, it is important that response to challenge is thorough and yet restricted enough so that normal tissue is not damaged during an immune response. When this response becomes uncontrolled, mucosal damage may occur, for instance in inflammatory bowel disease (IBD) (Shanahan & Targan, 1987), or coeliac disease (Kagnoff *et al.*, 1984). Organ culture studies of human fetal jejunum have shown that T cell activation can lead to local tissue injury (MacDonald & Spencer, 1988). Mitogen stimulation or direct T cell activation using monoclonal antibodies to the CD3 component of the T cell receptor can induce villous atrophy (epithelial injury) and crypt cell hyperplasia (MacDonald & Spencer, 1988).

Many different cytokines are produced during an immune response and these cytokines react with other cells of the immune system, and also affect the functions of non-immune cells, such as epithelial cells (Selby *et al.*, 1983; McDonald & Jewell, 1987). There is increased MHC class II expression on epithelial cells overlying inflamed areas in IBD tissues (Selby *et al.*, 1983), which can be induced *in vitro* by recombinant cytokines, such as interferon-gamma (IFN-γ) and/or tumour necrosis factor-alpha (TNF-α) (Selby *et al.*, 1983; McDonald & Jewell, 1987).

The goal of this study was to establish whether cytotoxic cytokines can be released *in vitro* by triggering isolated colonic T cells, and what cytokine interactions are required for killing of human colonic epithelial cells.

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MATERIALS AND METHODS

Target cells and culture media

The target cell lines K562 (a human myelogenous leukaemia line) and HT-29 (a human colon carcinoma line), were obtained from American Type Culture Collection, Rockville, MD. The murine fibroblast cell line, L929, was obtained from Dr G. A. Granger, University of California, Irvine. The K562 and L929 cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 (Irvine Scientific, Santa Anna, CA) supplemented with 10% fetal calf serum (FCS; from Irvine Scientific) and 3% FCS, respectively. The HT-29 cell line was cultured in McCoy's medium 5A supplemented with 10% FCS.

Monoclonal antibodies

Monoclonal antibodies anti-T8 (anti-CD8), anti-T4 (anti-CD4), and anti-T3 (anti-CD3) were purchased from Coulter Immunology (Hialeah, FA). Monoclonal antibodies anti-TNF-α and anti-IFN-γ were purchased from Olympus Immunochemicals (Lake Success, NY). Anti-TNF-β was purchased from Endogen (Boston, MA).

Recombinant cytokines

Recombinant human TNF-α and IFN-γ were purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant human TNF-β was purchased from R & D Systems (Minneapolis, MN).

Purification of LPL

Intestinal specimens were obtained from patients undergoing surgical resection of the colon at the University of California, Los Angeles, Center for the Health Sciences. The entire study was approved by the university's Human Subject Protection Committee. In this study, all tissue specimens were taken from an uninvolved area of colon from patients with colonic carcinoma. LPL were isolated using a technique modified from that originally described by Bull & Bookman (1977), as detailed previously (Shanahan, Brogan & Targan, 1987). Isolated LPL were further separated on Ficoll–Hypaque gradients (Böyum, 1968). In some experiments, purified populations of lymphocyte subsets were obtained by a 'panning' procedure using monoclonal antibodies directed against cell surface antigens (Wysoki & Sato, 1978). The LPL were resuspended in medium at 10^7 cells/ml and then incubated with monoclonal antibodies (0·25 μg/10^6 cells) for 60 min at 4°C. The cells were washed, resuspended in medium at 5×10^6 cells/ml, and 30×10^6 cells were added to 100-mm plastic Petri dishes (Falcon). These dishes were previously coated with 6 ml of purified goat $F(ab')_2$ anti-mouse IgG Fc (Jackson Laboratories, Avondale, PA) (20 μg/ml in 0·05 M Tris, pH 9·5) for 2 h at room temperature and then washed three times with medium. After 90 min of incubation at 4°C, the non-adherent cells were removed by gentle swirling and aspiration. The adherent cells were gently washed twice and then removed by vigorous washing.

Immunofluorescent staining

LPL (5×10^5) were suspended in phosphate-buffered saline (PBS) plus 2% FCS and 0·1% sodium azide (PBS–FCS) to a total volume of 100 μl, and phycoerythrin (PE) or red dye 1 (RD-1) and/or FITC-conjugated monoclonal antibodies (10 μl/test for Becton-Dickinson monoclonals, and 2·5 μl/test for Coulter monoclonals) were added for 30 min at 4°C. For panning-purified or complement-depleted cell populations, staining was done indirectly. Monoclonal-antibody-treated cells were washed and 0·1 μg of dichlorotriazinyl amino fluorescein-conjugated-purified $F(ab')_2$ goat anti-mouse IgG (H&L) (Jackson) was added for 30 min at 4°C. For direct, two-colour staining, control cells were stained with fluorochrome-conjugated, isotype-matched mouse immunoglobulin. For indirect staining, unconjugated, isotype-matched mouse immunoglobulin monoclonal-antibody-treated cells were stained with the secondary, fluorochrome-conjugated antibody. Stained cells were washed twice with PBS–FCS, resuspended in PBS, and fixed with 1% paraformaldehyde in PBS.

Two-colour flow cytometry

Analysis of lymphocyte subsets was performed using an EPICS 541 Flow Cytometer (Coulter). Red and green fluorochromes were excited using 400 mW of 488 nm light from an argon laser. Fluorescent emission was collected by means of a focusing lens placed at 90° relative to the laser–stream intersection. Forward angle light scatter was collected by a pickup lens (photodiode) in the laser light path. A 488 nm dichroic filter was placed at a 45° angle to the light path to separate the 90° scatter from the red and green fluorescence. A 488 nm long pass laser blocking filter was placed after the dichroic filter. A second splitting filter was placed 45° relative to the light path to separate red and green fluorescent emissions. A 590 nm long pass filter was placed in front of the red photomultiplier tube, and a 530 nm short pass filter was placed in front of the green photomultiplier tube. The amplitudes of the electrical signals were converted from analog to digital by means of the Analog to Digital Converter. The data were analysed and histograms displayed by means of the Multiparameter Data Acquisition and Display System. Dead cells, erythrocytes, platelets, and monocytes were excluded from the analysis by gating (forward angle and 90° light scatter) on the lymphocyte population.

Soluble factor lytic activity

Soluble factor lytic activity was determined using L929 fibroblasts as target cells in an 18-h ^{51}Cr release assay as described previously (Deem & Targan, 1984), or HT-29 carcinoma cells as targets in a 48-h ^{111}In release assay. Cytotoxicity tests were performed in 96-well, flat-bottomed microplates (Costar) in a total volume of 200 μl. L929 target cells were labelled with 200 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) for 1 h at 37°C, and were washed three times; and 10^4 target cells were plated in each microtitre well. HT-29 target cells were labelled with 100 μCi ^{111}In oxime (Amersham, Arlington Heights, IL) for 30 min at 37°C, and were washed three times; and 10^4 target cells were plated in each microtitre well. The target cells were allowed to adhere 18 h, then washed, and medium replaced by RPMI 1640 with 3% FCS (L929) or McCoy's medium with 10% FCS (HT-29), and diluted supernatants were added to a total volume of 200 μl/well, and incubated. A wide range of dilutions was used to generate cytotoxicity curves. Results were expressed as % cytotoxicity as follows:

$$\frac{\text{Experimental} - \text{Spontaneous release}}{\text{Total} - \text{Spontaneous release}} \times 100$$

Spontaneous release for these assays was always less than 25%

for the 18-h ^{51}Cr release assay and less than 20% for the 48-h ^{111}In release assay. Lytic units (LU) were calculated from a semi-logarithmic curve, plotting the natural logarithm of the reciprocal dilution factor *versus* % cytotoxicity (based on the formula $y = a + b*ln(x)$, where x is the dilution factor, y is the percent cytotoxicity, a is the y intercept, and b is the slope). One LU was defined as the reciprocal dilution factor required to induce a given % cytotoxicity from 10^4 target cells. R values were consistently 0·95 or greater for these titration curves. Data acquisition and reduction were performed using the Gamma Master program for Macintosh computers, developed by one of us (R.L.D.).

Soluble factor cytostatic activity

Soluble factor cytostatic activity was determined using HT-29 carcinoma cells as targets in a 72-hr ^3H-thymidine incorporation assay. These tests were performed in 96-well, flat-bottomed microplates (Costar) in a total volume of 200 μl. HT-29 target cells (2×10^3) were plated in each microtitre well, diluted supernatants were added to a total volume of 200 μl/well, and incubated for 48 h at 37°C. Wells were pulsed with 40 μCi ^3H-thymidine (New England Nuclear) for 24 h at 37°C. Wells were harvested using a PHD™ Cell Harvester (Cambridge Technology, Cambridge, MA) and counted in 7-ml mini-vials on a Spectral 1219 liquid scintillation counter (LKB, Gaithersberg, MD). Percent inhibition of growth (% cytostasis) was calculated for six serial dilutions of supernatants by comparison to wells containing untreated HT-29 cells. Cytostatic units were calculated from a semi-logarithmic curve plotting the natural logarithm of the reciprocal dilution factor *versus* % cytostasis. One cytostatic unit was defined as the reciprocal dilution factor required to induce 30% cytostasis from 2500 target cells.

Statistical analysis

Tests for significance of differences were done using Student's *t*-test.

RESULTS

LPL incubated with anti-CD3 monoclonal antibody and K562 target cells produce factors cytostatic for HT-29 cells and cytotoxic for L929 cells

In order to examine the possible role of LPL in immune-mediated injury of the colon, LPL were incubated under various conditions for 18 h and supernatants were harvested. These supernatants were tested for their ability to inhibit the growth of HT-29 target cells in a 72-h ^3H-thymidine incorporation assay. LPL from normal colonic mucosa, when incubated alone or with target cells only, did not produce cytostatic supernatants (Fig. 1). However, when LPL were incubated with anti-CD3 or anti-CD3 plus K562 target cells, cytostatic supernatants were produced (Fig. 1). Incubation of LPL with both anti-CD3 and target cells produced approximately three times the level of cytostasis, compared with incubation of LPL with anti-CD3 alone ($P < 0·05$) (Fig. 1). These LPL supernatants also contained cytotoxic cytokines, as shown by their ability to lyse the murine fibroblast line L929 (Fig. 2). Cytotoxic cytokines were produced in the same proportion as cytostatic cytokines shown in Fig. 1.

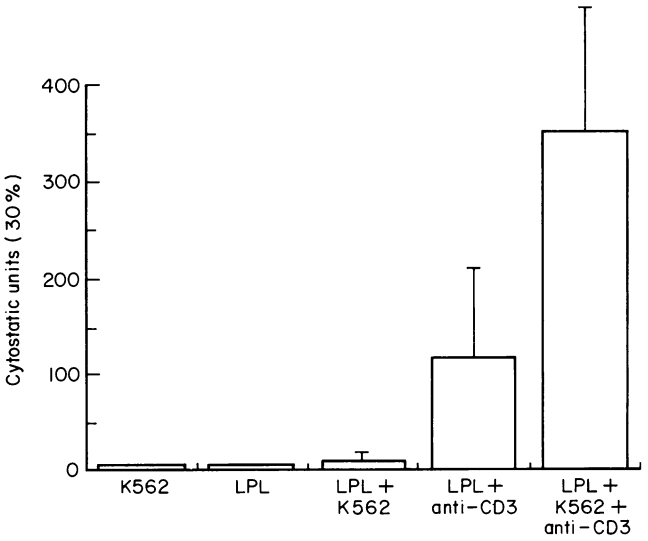

Fig. 1. Effect of supernatants derived from lamina propria lymphocytes (LPL) upon growth of HT-29. Cytostatic units were calculated as indicated in Materials and Methods. Bars represent s.e.m. from four LPL preparations.

LPL incubated with anti-CD3 monoclonal antibody and K562 target cells produce factors cytotoxic for HT-29 cells

Microscopic examination of 72-h HT-29 growth experiments suggested that LPL supernatants were producing cytostasis at least partially by killing the HT-29 cells. Preliminary experiments demonstrated that LPL supernatants produced some killing of HT-29 targets when incubated for 24 h in a ^{51}Cr release assay. Longer incubations in a ^{51}Cr release assay were impossible because of high spontaneous release, so a 48-h ^{111}In release assay was used to examine killing of HT-29 targets. There was little or no cytotoxicity when HT-29 targets were incubated in supernatants generated from the incubation of LPL alone, with K562 target cells only, or with anti-CD3 only (Fig. 3). However,

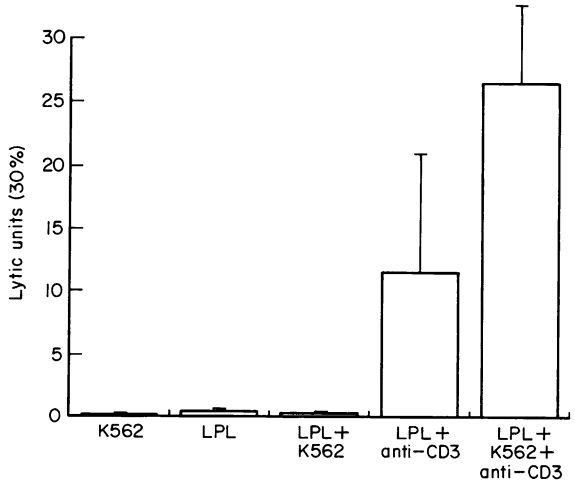

Fig. 2. Effect of supernatants derived from lamina propria lymphocytes (LPL) upon lysis of L929. Lytic units were calculated as indicated in Materials and Methods. Bars represent s.e.m. from five LPL preparations.

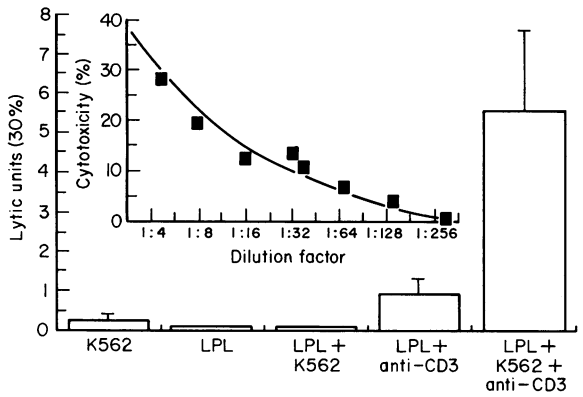

Fig. 3. Killing of HT-29 by supernatants derived from lamina propria lymphocytes (LPL). Results are expressed as lytic units and were calculated as indicated in Materials and Methods. Bars represent s.e.m. from six LPL preps. Inset: Sample titration of LPL supernatant (LPL incubated with K562 targets and anti-CD3 antibody) showing % cytotoxicity at different dilutions of supernatant.

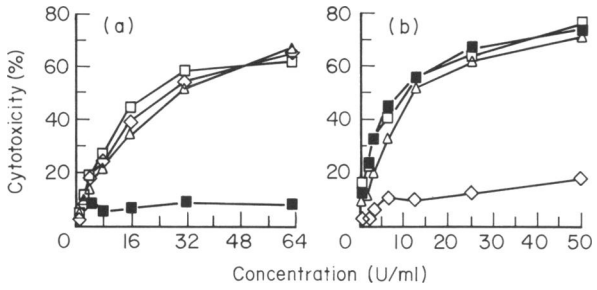

Fig. 4. Specificity of anti-cytokine antibodies. (a) Antibodies (1 μg/ml) and dilutions of TNF-β were added to L929 targets for 18 h at 37°C; (b) antibodies (1 μg/ml) and dilutions of TNF-α were added to L929 targets for 18 h at 37°C; □, no antibodies; ■, anti-TNF-β; ◇, anti-TNF-α; △, anti-IFN-γ.

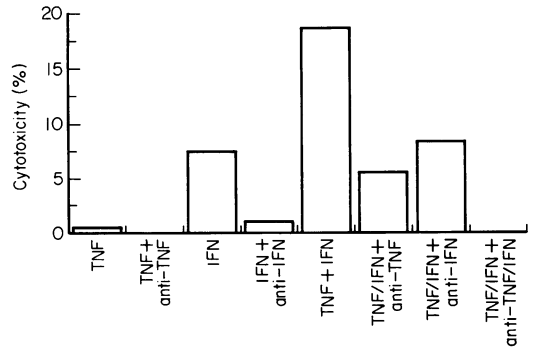

Fig. 5. Effect of anti-cytokine antibodies upon killing of HT-29 by recombinant cytokines. TNF-α (10 U/ml) and/or IFN-γ (100 U/ml) were added to ^{111}In-labelled HT-29 cells for 48 h at 37°C in the presence and absence of 1 μg/ml anti-cytokine antibodies.

incubation of HT-29 with supernatants generated from LPL with K562 targets and anti-CD3 produced high killing (up to 32% cytotoxicity at a dilution of 1:4, Fig. 3, inset) ($P < 0.001$ compared with all other groups).

Anti-cytokine antibodies demonstrate specific neutralization of cytokine action

Specific neutralizing monoclonal antibodies were used to determine what cytokines were present in the LPL supernatants. Recombinant cytokines were used to test the specificity of the anti-cytokine antibodies. Recombinant TNF-α and TNF-β killed L929 target cells when incubated for 18 h at 37°C (Fig. 4). Addition of 1 μg/ml anti-TNF-β nearly completely inhibited cytotoxicity by TNF-β ($P < 0.001$), although anti-TNF-α and anti-IFN-γ had no effect ($P > 0.05$) (Fig. 4). Likewise, addition of 1 μg/ml anti-TNF-α nearly completely inhibited cytotoxicity by TNF-α ($P < 0.001$), although anti-TNF-β and anti-IFN-γ had no effect ($P > 0.05$) (Fig. 4). Anti-IFN-γ was shown to inhibit the cytotoxic effects of IFN-γ on HT-29 target cells (Fig. 5), although anti-TNF-α and anti-TNF-β had no effect (data not shown). These results demonstrated that these antibodies were specific in their anti-cytokine inhibitory effects.

TNF-α and IFN-γ synergistically kill HT-29 target cells

Recombinant cytokines, which were likely to be produced upon interaction of LPL with target cells, were tested for their ability to kill HT-29 targets. TNF-α by itself had little effect upon killing of HT-29 (Fig. 5). IFN-γ did kill HT-29 targets when incubated for 48 h (Fig. 5). When these cytokines were combined, killing was enhanced (Fig. 5). Addition of antibodies against either cytokine alone partially inhibited this killing, and addition of both anti-cytokine antibodies nearly completely inhibited cytotoxicity (Fig. 5). These results suggested that although IFN-γ alone could produce some killing, optimal lysis of HT-29 occurred with a combination of IFN-γ and TNF-α. Therefore IFN-γ and TNF-α could be responsible for the cytotoxicity observed when LPL supernatants were incubated with HT-29 target cells.

Anti-TNF-α and anti-IFN-γ inhibit killing of HT-29 target cells by LPL supernatants

Anti-cytokine antibodies were added to LPL supernatants to determine which cytokines were present in the LPL supernatants. Addition of either anti-TNF-α or IFN-γ resulted in nearly complete inhibition of HT-29 killing ($P < 0.001$) by supernatants produced from the incubation of LPL with K562 targets and anti-CD3 antibody (Fig. 6), demonstrating that both cytokines were present in the LPL supernatants and that the presence of both cytokines was required for optimal killing of HT-29.

CD4$^+$ and CD8$^+$ LPL produce TNF-α when incubated with K562 targets and anti-CD3 antibody

Since this was the first demonstration of TNF-α production from activated mucosal T cells, it was important to confirm which T cell subsets were responsible. Both CD4$^+$ (>95% pure) and CD8$^+$ (>90% pure) LPL were separated by indirect panning. Both subpopulations produced TNF-α when stimulated with K562 targets and anti-CD3, although CD4$^+$ LPL produced larger quantities of TNF-α (Fig. 7). Neither subpopu-

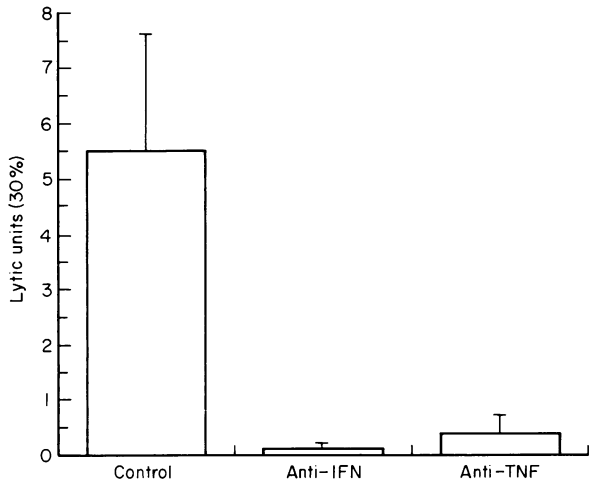

Fig. 6. Effect of anti-cytokine antibodies upon killing of HT-29 by supernatants derived from lamina propria lymphocytes (LPL). Supernatants were added to ^{111}In-labelled HT-29 cells for 48 h at 37°C in the presence and absence of 1 μg/ml anti-cytokine antibodies. Lytic units were calculated as indicated in Materials and Methods. Bars represent s.e.m. from six LPL preparations.

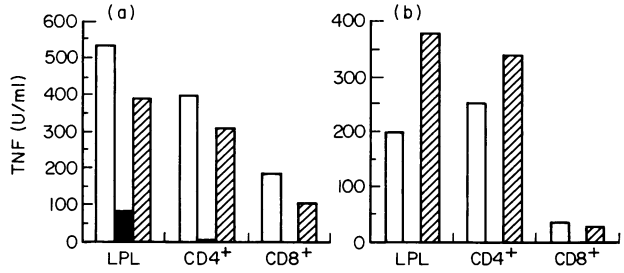

Fig. 7. Lamina propria lymphocyte (LPL) subpopulations responsible for generation of cytokines. LPL-derived supernatants were added to ^{51}Cr-labelled L929 cells for 18 h at 37°C. Lytic units were calculated as indicated in Materials and Methods, and adjusted to represent TNF units by comparison to a recombinant TNF standard. □, no antibody added; ■, 1 μg/ml anti-TNF-α added; ▨, 1 μg/ml anti-TNF-β added. LPL supernatants from two colon resections are shown (a and b).

lation produced much if any TNF-β, since anti-TNF-β antibody had little effect upon killing of L929 targets (Fig. 7).

DISCUSSION

This study was designed to examine how mucosal T cells contribute to the damage caused to epithelial cells during an immune response. Mucosal T cells were polyclonally activated by incubation with anti-CD3 monoclonal antibody in combination with cross-linking via an Fc receptor-expressing target cell (K562). This treatment resulted in secretion of cytokines that were cytostatic to the human epithelial colon line, HT-29. Subsequent experiments demonstrated that these supernatants also were cytotoxic to the murine fibroblast line, L929 and HT-29. Little or no spontaneous secretion of these cytokines occurred and incubation of LPL with anti-CD3 alone resulted in less secretion of cytotoxic/cytostatic cytokines, compared with incubation with anti-CD3 and K562 targets. These results suggest that cross-linking of the CD3 receptor is probably required for optimal triggering of cytokine release.

Specific anti-cytokine monoclonal antibodies were used to determine what cytokines were produced by the triggered LPL. TNF-β, which one might expect to be produced by cytotoxic T cells, is either present in low quantities or not at all, as shown by the inability of anti-TNF-β antibody to neutralize supernatant cytotoxicity against L929 (Fig. 7). However, either anti-TNF-α or anti-IFN-γ inhibited HT-29 cytotoxicity induced by LPL supernatants, demonstrating that both cytokines were secreted by the LPL and both were required for optimal killing of HT-29. Further evidence was seen in experiments using recombinant TNF-α and IFN-γ, which also demonstrated that both cytokines were required for optimal killing of HT-29.

Recent studies have shown that T cell lines and T cells from the peripheral blood produce TNF-α and TNF-β, when activated through the CD3 receptor (Hara & Fu, 1985; Weiss *et al.*, 1986; Steffen, Ottmann & Moore, 1988; Sung *et al.*, 1988; Thompson *et al.*, 1989). These studies used either anti-CD3 in combination with IL-2 or phorbol 12-myristate 13-acetate (PMA). This study has shown that mucosal T cells also produce TNF-α but not TNF-β when activated through the T cell receptor with anti-CD3 antibody in combination with K562 target cells. In addition, both CD4$^+$ and CD8$^+$ lamina propria T cells produced TNF-α when activated.

TNF-α and IFN-γ have been shown to be capable effector molecules in immune destruction of self tissues (Sung *et al.*, 1988; Campbell, Iscaro & Harrison, 1988; Thompson *et al.*, 1989). Individually or in combination, IFN-γ or TNF-α are capable of destroying target tissues of known autoimmune responses. This has been demonstrated recently in an animal model of diabetes where a combination of IFN-γ and TNF-α destroyed isolated β-islet cells *in vitro* (Campbell *et al.*, 1988). In addition, TNF-α has been identified in lesions from brain tissues from patients with multiple sclerosis (Hofman *et al.*, 1989). Analysis of the specificity of these cytotoxic/cytostatic lymphokines has demonstrated that not all cells are equally sensitive to their effects (Sugarman *et al.*, 1985). Normal colonic epithelial cells may or may not have the same sensitivity to these cytokines as colonic cell lines. The ability of IFN-γ to upregulate a variety of cellular receptors including those for TNF-α suggests one mechanism responsible for synergism between these two cytokines. Sensitivity of targets to TNF-α and IFN-γ may be related to expression of certain antigens or receptors on their cell surface or effects of viral products on cellular metabolism. This has been demonstrated most recently in the destruction of virally infected human targets by CTL-released lymphokines (Paya *et al.*, 1989) and the enhanced lysis of fibroblasts infected with adenovirus by TNF-α (Duerksen-Hughes, Wold & Gooding, 1989). Thus villous cell atrophy and crypt cell hyperplasia seen following polyclonal T cells activation in fetal jejunum organ culture (MacDonald & Spencer, 1988) could reflect differing responses of these two cell types to the released cytokines.

These results demonstrate that colonic mucosal T cells, triggered through the T cell receptor, produce both TNF-α and IFN-γ *in vitro*. A combination of both of these cytokines is required for killing the human colonic epithelial target cells line, HT-29. This study suggests that human mucosal T cells may be involved in indirect, cytokine-mediated damage to colonic mucosa, during immune reactions.

ACKNOWLEDGMENTS

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