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Presence of tumour necrosis factor or a related factor in human basophil/mast cells

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SUMMARY

The observation that mast cell products and cachectin/tumour necrosis factor (TNF) mediate similar responses suggested an investigation of cultured human basophil/mast cells for production of TNF. Using *in situ* hybridization and the avidin–biotin-complex (ABC) immunoperoxidase method, we have demonstrated the presence of TNF mRNA in the cytoplasm and TNF protein in the granules of individual human basophil/mast cells. The production of TNF by these cells could explain many of their reported functions.

INTRODUCTION

Mast cells are distributed widely throughout human tissues, most abundantly in the skin and the gastrointestinal mucosa. They are characterized by their metachromatic granules and synthesis of a number of recognized pharmacologically potent mediators. Some of these mediators, such as histamine, heparin, eosinophil chemotactic factor, platelet-activating factor and proteolytic enzymes (Lee *et al.*, 1985), are produced and stored in the granules, to be released on stimulation. Mast cells are also able to synthesize arachidonic acid metabolites that are mediators of allergy and inflammation (Lee *et al.*, 1985).

The role of mast cells in the allergic response and anaphylaxis is well understood. Mast cells are also involved in a number of processes other than allergy and anaphylaxis, but their role is not as well defined (Lee *et al.*, 1985). These processes include bone remodelling (McKenna & Frame, 1985), stimulation of fibroblast proliferation (Atkins *et al.*, 1985; Claman 1985), stimulation of collagenase and prostaglandin E_2 production by cultures of rheumatoid synovial cells (Yoffe, Taylor & Woolley, 1984), activity in parasitic infections (Capron *et al.*, 1978; Justus & Morakote, 1981), and cytotoxicity against tumour cells *in vitro* (Farram & Nelson, 1980; Henderson *et al.*, 1981). Cachectin/tumour necrosis factor (TNF), a multifunctional cytokine (Beutler & Cerami, 1987), is now known to mediate many of the biological processes in which mast cells are also involved (Beutler & Cerami, 1987; Bertolini *et al.*, 1986; Vilcek *et al.*, 1986; Dayer, Beutler & Cerami, 1985; Silberstein & David, 1986; Carswell *et al.*, 1975; Sugarman *et al.*, 1985). TNF production by mast cells could help to explain their role in these processes.

The study of human basophil/mast cells has long been hampered by their biological heterogeneity (Lee *et al.*, 1985), difficulty in isolation and absence of a consistent and successful culture system. Recently, we have been able to culture basophil/mast cells from normal human bone marrow precursors in medium conditioned by mitogen-stimulated human splenocytes (Abboud *et al.*, 1986). We also characterized these cells by cytochemical, immunochemical and ultrastructural studies (Abboud *et al.*, 1986). Their granules stained metachromatically with toluidine blue and reacted with alcian blue after competitive alcian blue-safranin staining (Pretlow & Cassady, 1970). The cells contained histamine and had surface receptors for IgE. Electron microscopy of these cells revealed features associated with mast cells, e.g. typical cytoplasmic granules containing fibrillar vesicular and electron dense material. In some granules early whorl formation was evident.

MATERIALS AND METHODS

Cell cultures

Low density human bone marrow cells were obtained by density gradient sedimentation with Ficoll–Hypaque (density 1·007 g/ml; Pharmacia, Piscataway, NJ) and depleted of adherent cells by incubation for 2 hr in Corning tissue culture flasks at 37° in humidified 5% CO_2 air. Low density non-adherent cells were cultured at 1×10^6/ml in McCoy's 5A medium (Gibco, Chagrin Falls, OH) supplemented with 10% fetal bovine serum (FBS) and 10% spleen cell-conditioned medium. A total of 10^7 cells were cultured in each upright 25 cm^2 Corning tissue culture flask. The cultures were incubated at 37° in humidified 5% CO_2 air. Fresh medium was added weekly after demidepopulation.

Conditioned medium for the human basophil/mast cell cultures was prepared by culturing human spleen lymphocytes

Abbreviations: G-CSF, granulocyte colony-stimulating factor; TNF, tumour necrosis factor.

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(from patients undergoing splenectomy for chronic myeloic leukaemia (CML), chronic lymphatic leukaemia (CLL), Hodgkin's disease and solid tumours) at 1×10^6 per ml of RPMI-1640 medium supplemented with 5% FBS and 2 μg/ml concanavalin A (Con A) or 1% phytohaemagglutin (PHA). Cultures were incubated at 37° in humidified 5% CO_2 air; supernatants were collected after 7 days, filtered and stored at 4°.

Murine FI mast cells (Yung & Welte, 1985) were grown in RPMI with 10% FBS and 10% LBRM-33 cell-conditioned medium. LBRM-33 cells are derived from a radiation-induced thymic lymphoma in a B10.Br mouse and were stimulated by 2 μg/ml Con A.

5637 bladder carcinoma cells and HL-60 cells were cultured in RPMI with 10% FBS. HL-60 cells were induced by 20 ng/ml phorbol myristate acetate (PMA) for 48 hr.

In situ hybridization

In situ hybridization was performed as described by Pardue (1985). Briefly, hybridization mixtures (^{35}S-labelled TNF cDNA (Cetus, Emeryville, CA) in 4 × SSC; Maniatis, Fritsch & Sambrook, 1982; 50% formamide, 0·5 mg/ml salmon sperm DNA; Sigma) were applied to the cytocentrifuge smears after toluidine blue staining. Human TNF cDNA (Wang *et al.*, 1985) was ^{35}S-labelled by the method of primer extension (Feinberg & Vogelstein, 1984). After hybridization in moist chambers for 24 hr at 37°, slides were rinsed extensively in 4 × SSC, 2 × SSC, 1 × SSC, and dehydrated. Autoradiography was performed with Kodak NTB-2 emulsion, with exposure up to 4 weeks. As a control for unspecific binding of the cDNA probe, parallel slides were hybridized with ^{35}S-labelled human G-CSF cDNA (kindly provided by Dr L. M. Souza, Amgen, CA).

Northern blot analysis

Total cellular RNA of mouse FI mast cells, human PMA-induced HL-60 cells and human 5637 bladder carcinoma cells was prepared by the guanidinium isothiocyanate-caesium chloride method (Chirgwin *et al.*, 1979). Poly A^+ RNA, purified from the total RNA by oligo (dT) column, was analysed by electrophoresis in 1·1% agarose formaldehyde gels, followed by Northern blot transfer to Nytran membrane (Maniatis *et al.*, 1982). The gel was stained with ethidium bromide to confirm that approximately equivalent amounts of RNA were loaded in each gel lane. RNA blot was prehybridized at 42° for 16 hr in 50% formamide, 6 × SSC, 5 × Denhard's solution (Maniatis *et al.*, 1982, 1% SDS and 250 μg/ml salmon sperm DNA. Blot was hybridized in the same buffer with 2×10^6 c.p.m. per ml of human TNF cDNA (Wang *et al.*, 1985) that was ^{32}P-labelled using the method of primer extension (Feinberg & Vogelstein, 1984). After hybridization, the blot was washed twice in 6 × SSPE (Maniatis *et al.*, 1982), 0·2% SDS at room temperature for 20 min, twice in 1 × SSPE, 0·5% SDS at 37° for 20 min, and finally in 0·1 × SSPE, 1% SDS at 50° for 1 hr. The blots were exposed to Kodak XRP-1 film with an intensifying screen at −70°.

Cytochemical staining

Cytocentrifuge preparations were air dried and fixed-stained for 15 min in 1% toluidine blue-O in absolute methanol, followed by a wash in distilled water, air drying and mounting in Permount. Similar smears were fixed and stained for butyrate esterase reactivity by the technique of Li, Yam & Crosby (1972), and by the May–Grünwald technique.

For immunoperoxidase staining by the ABC technique, air-dried cytocentrifuge preparations were fixed in cold (−20°) acetone. To eliminate endogenous peroxidase reactivity smears were placed in a 1% solution of H_2O_2 in absolute methanol for 30 min, followed by a wash with distilled water. Specimens were then placed in 0·05% aqueous saponin (Sigma, St Louis, MO) for 10 min, to enhance penetrability of reagents, and similarly washed, followed by immersion in PBS (pH 7·4) for 5 min and blocking with normal goat serum (Vectastain Kit No. 4001, Vector Laboratories, Burlingame, CA) for 20 min to block surface Fc receptors, and the excess drained from the slides without subsequent washing. Slides were incubated overnight at 4° in primary antibody (rabbit anti-recombinant human TNF, IgG complexes, kindly provided by Dr B. Rubin, or polyvalent antibodies against homogenous TNF, from Endogen, Boston, MA), in a dilution of 1 : 400 and washed twice with two changes of PBS (5 min each). As negative controls, parallel slides were treated with PBS or FBS instead of the TNF antiserum. Secondary antibody (goat anti-rabbit IgG) was applied according to Vectastain kit instruction for 1 hr at room temperature, followed by washes as above. Avidin–biotin complex was applied per kit instructions for 30 min, followed by PBS washes as above, and development of reaction product in a solution containing 0·5% Triton X-100, 0·05% DAB (Sigma) and 0·01% H_2O_2, checking reactivity with a microscope. At maximum reaction (5–6 min), the slides were transferred to PBS, washed for 5 min in distilled water, and counterstained in Harris' haematoxylin for 1 min, washed, dehydrated through graded alcohols, cleared in xylene and mounted in Permount.

Assay for TNF in culture supernatants

Biologically active TNF in culture supernatants was detected by the MTT colorimetric assay (Green, Reade & Ware, 1984). Briefly, cells of the fibrosarcoma line WEHI 164 (Walter and Eliza Hall Institute, Melbourne, Australia), which are highly sensitive to TNF (Okuno *et al.*, 1986), were used as target cells. Cells (5×10^3 cells/well) were cultured in 96-well plates (Falcon, Becton-Dickinson, Oxnard, CA) at 37° in 100 μl RPMI medium with 10% FBS. One-hundred-microlitre samples of supernatants from the long-term bone marrow cultures were added to the wells in the presence or absence of polyvalent antibodies against homogenous human TNF (Endogen, Boston, MA). After 44 hr in culture, a tetrazolium dye [MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-dimethyl tetrazolium bromide; Sigma] was added to the culture; 4 hr later, the amount of blue formazan deposits synthesized by the living cells was quantified by a microplate spectrophotometer. In this assay, the OD values are inversely related to the degree of cytotoxic activity mediated by TNF. Internal standards were included in each experiment. The antibodies were not toxic by themselves and did not cross-react.

RESULTS

Human basophil/mast cells from the long-term bone marrow cultures were examined in cytocentrifuge smears that were prepared weekly. The cell population consisted of small round granular cells and larger non-granular cells. The small round granular cells demonstrated round or slightly irregular, non-lobulated, eccentric nuclei and appeared basophilic, after staining by the May–Grünwald Giemsa technique, but were not reactive for butyrate esterase. These cells were confirmed

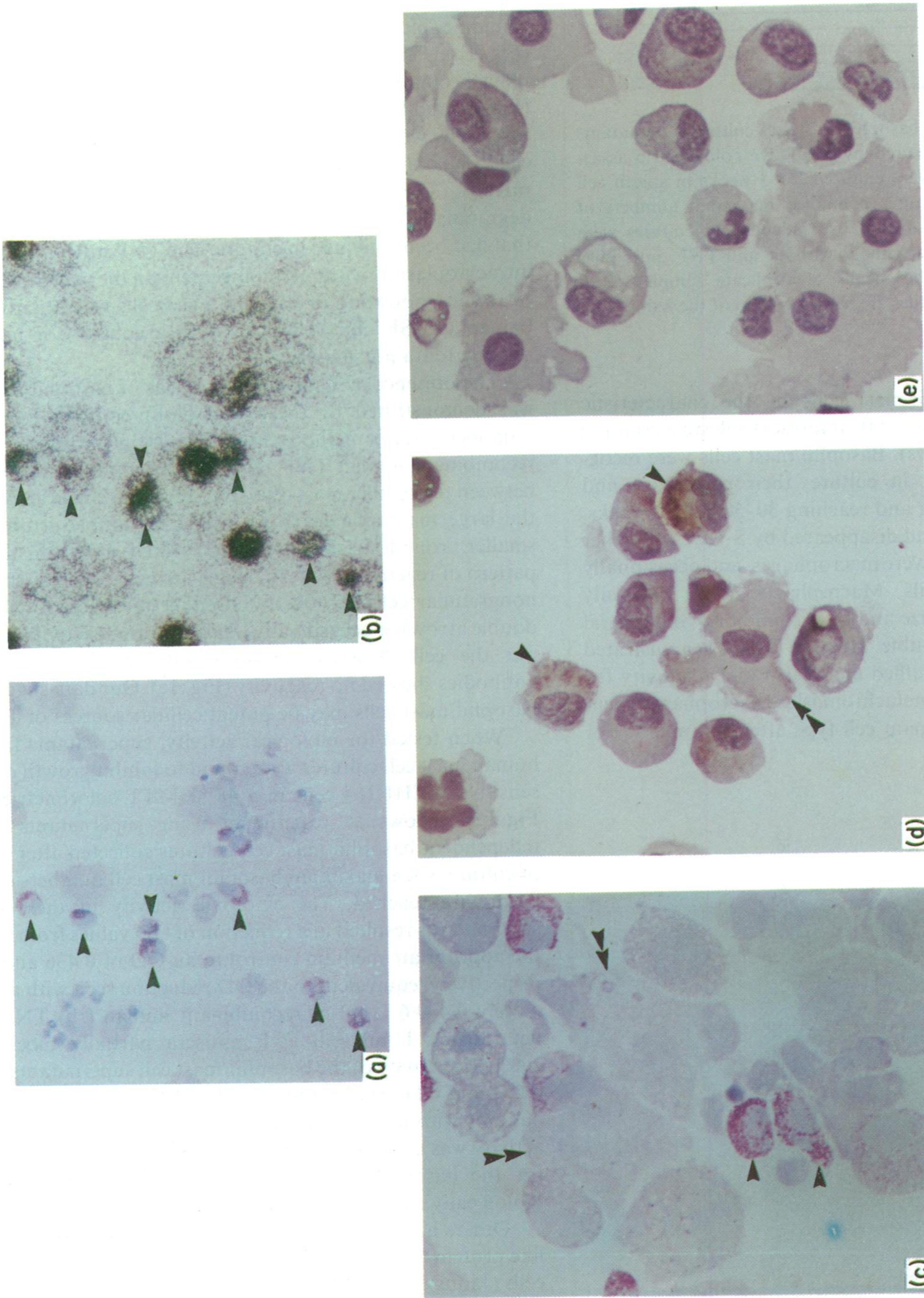

Figure 1. Detection of TNF mRNA and protein in cultured human basophil/mast cells. (a) Toluidine blue-stained cytosmear from 3-week-old culture, ×100. Arrows point to toluidine-stained basophil/mast cells. (b) The same cells after *in situ* hybridization (arrows) with ^{35}S-labelled TNF cDNA probe (Wang *et al.*, 1985) after toluidine blue staining, ×100. (c) Toluidine blue-stained cytosmear from similar culture, ×250. (d) Cultured basophil/mast cells after ABC immunoperoxidase reaction using antiserum against recombinant human TNF, ×250. Single arrows: granular reactivity pattern in basophil/mast cells; double arrows: large nongranular macrophage. (e) ABC immunoperoxidase reaction of cultured basophil/mast cells without the TNF-specific antibodies as negative control, ×250.

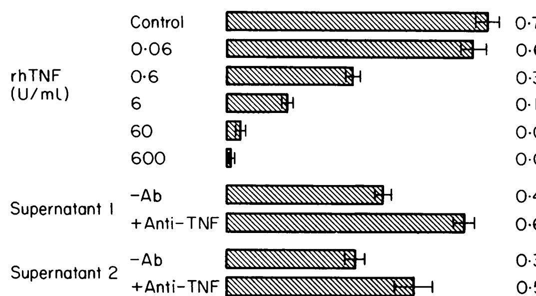

Figure 2. Cytotoxicity of the basophil/mast cell culture supernatants against WEHI 164 cells measured by MTT-dye colorimetric assay. Human basophil/mast cells were cultured for 3 weeks in spleen cell conditioned medium; the cultures contained maximum numbers of basophil/mast cells. Supernatants of two independent cultures were added to the WEHI 164 cells with or without anti-TNF antibodies (50 U/ml). Data represent the means of triplicate samples ±SE indicated by bars. The OD values are shown on top of the bars.

cytochemically as basophil/mast cells by the characteristic toluidine blue metachromasia of their intracytoplasmic granules (Fig. 1a and c, single arrows). Basophil/mast cells were recognized after the first week in culture, their percentage and absolute number increasing and reaching 30–50% by 3 weeks, after which they declined and disappeared by 5–6 weeks. Other cells present in the cultures were macrophages, and occasionally neutrophils and eosinophils. Macrophages could be easily recognized by their larger size and their round or ovoid central nuclei (Fig. 1a and c, double arrows). They demonstrated smooth, agranular, often ruffled cytoplasm and reactivity for butyrate esterease, but no metachromasia. Macrophages eventually became the predominant cell type after 5–6 weeks. For these experiments, 2–3-week-old cultures containing maximum numbers of basophil/mast cells were used.

To demonstrate the presence of TNF mRNA in human basophil/mast cells, cytospin preparations from the long-term bone marrow cultures were stained with toluidine blue, followed by *in situ* hybridization using a ^{35}S-labelled TNF cDNA probe. Figure 1a and b represent a single field after staining with toluidine blue (Fig. 1a), followed by *in situ* hybridization (Fig. 1b). The very same basophil/mast cells, identified by their metachromatic granules, were found to contain TNF mRNA (Fig. 1a and b, single arrows). In our preparation, all basophil/mast cells were found to contain TNF mRNA. Occasionally, cells, lacking metachromatic granules, also expressed TNF mRNA. These cells could be basophil/mast cells that had degranulated during the preparation, or small macrophages that had been activated in culture. The TNF mRNA was clearly intracytoplasmic without impingement on the nucleus (Fig. 1b). *In situ* hybridization of basophil/mast cells was negative when human G-CSF cDNA was used as a control for unspecific binding (data not shown).

The presence of TNF in the granules of basophil/mast cells was demonstrated by the avidin–biotin-complex (ABC) immunoperoxidase method (Fig. 1d), using antiserum against recombinant human TNF. A clear distinction could be made between the smaller mast cells (Fig. 1c and d, single arrows) and the large macrophages (Fig. 1c and d, double arrows). The smaller, round mast cells demonstrated a rough, granular pattern of reactivity (Fig. 1d, single arrows), whereas the larger nongranular cells demonstrated either no reactivity (Fig. 1d, double arrow) or occasionally weak uniform reactivity throughout the cell. Negative controls without the TNF-specific antibodies showed no reactivity (Fig. 1e). Our data suggest that basophil/mast cells may be potent cellular sources of TNF.

When tested for biological activity, supernatants from the human mast cell cultures were found to inhibit growth of TNF-sensitive WEHI 164 cells in a 48-hr MTT colorimetric assay. Figure 2 shows an experiment using supernatants of two independent basophil/mast cell cultures collected after 3 weeks of culture when maximum basophil/mast cell numbers (30% or more) are reached. The cytotoxic activity of these culture supernatants resulted in a reduction of OD values from 0·715 of the appropriate medium control to an OD of 0·436 and 0·353, respectively, equivalent to the OD reduction seen with approximately 0·4–0·6 U/ml of recombinant human (rh) TNF. Fifty neutralizing U/ml anti-TNF antisera partially blocked the cytotoxic activity of the basophil/mast cell supernatants (OD = 0·654 and OD = 0·515, respectively). TNF was undetectable in cultures with less than 10% basophil/mast cells. No cytotoxic activity was observed using the spleen cell-conditioned medium for the basophil/mast cell cultures. These data show that biologically active TNF was produced by cells in the culture.

Others have demonstrated the presence of TNF or a TNF-like protein responsible for natural cytotoxicity in murine mast cells (Jadus *et al.*, 1986; Okuno *et al.*, 1986; Young *et al.*, 1987). These investigators, however, did not demonstrate the presence of TNF mRNA in these cells. We looked for the production of TNF mRNA by Northern blot analysis in the mouse mast cell line FI, established as described earlier (Yung & Welte, 1985). Since human and mouse TNF show 80% homology at the amino acid level, the human TNF cDNA probe was used. The Northern blot showed TNF-specific mRNA prepared from FI

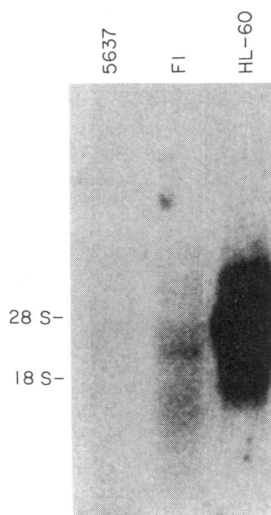

Figure 3. TNF mRNA production by cells of the mouse mast cell line FI. Northern blot analysis using 10 μg poly (A)$^+$ RNA per lane and ^{32}P-labelled TNF cDNA (Wang *et al.*, 1985) as a probe. Poly (A)$^+$ RNA from HL-60 cells grown for 48 hr in the presence of 20 ng/ml PMA was used as positive control. No TNF mRNA was detected in cells of the human bladder carcinoma line 5637 as negative control.

cells close to the region of 18 S rRNA (Fig. 3). mRNA from PMA-induced HL-60 cells was used as a positive control for TNF mRNA production; mRNA from 5637 bladder carcinoma cells served as a negative control (Fig. 3).

DISCUSSION

TNF is a potent cytokine produced by activated monocytes. It is known to have a wide spectrum of biological activities and to mediate a number of processes in which mast cells also play a role. We have demonstrated the presence of TNF mRNA and protein in cultured human basophil/mast cells. As these cells were found in cultures that contained other cell types the technique of *in situ* hybridization and immunocytochemistry were chosen to detect TNF mRNA and protein, respectively, in individual cells.

The presence of TNF mRNA was demonstrated in the same cells that could be characterized as basophil/mast cells by toluidine blue staining. Occasionally, toluidine blue-negative cells were found to contain TNF mRNA. These could be either degranulated basophil/mast cells or monocytes that had been activated in culture. Since different and incompatible fixation methods are required for the toluidine blue stain and immunocytochemistry, TNF protein could not be shown in the same cells that had been stained with toluidine blue. However, the immunoperoxidase-positive cells could be clearly distinguished from the macrophages by their smaller size, their granular pattern of immunoperoxidase reactivity in the cytoplasm and their nuclear morphology, which was identical to that of the toluidine blue-stained cells in the same culture. Biologically active TNF was demonstrated in the supernatants of the basophil/mast cell cultures. Since many macrophages, a well-known source of TNF, were present in these cultures, the presence of TNF in the culture supernatants can not be attributed solely to basophil/mast cells.

Young *et al.* (1987) have recently demonstrated the presence of a protein that is functionally similar to TNF in the granules of cultured and naturally occurring murine mast cells. This protein, however, had a molecular weight of 50,000 by immunoblot analysis under reducing conditions, which make it distinct from murine TNF with a molecular weight of 20,000 under these conditions. Others have shown that murine mast cells have cytotoxic functions that are probably mediated by TNF (Okuno *et al.*, 1986; Jadus *et al.*, 1986). We have demonstrated the production of TNF mRNA by these cells. The weakness of the band in the Northern blot with the FI cell RNA is probably due to the fact that FI cells represent an early stage in mast cell differentiation, or less likely, that a human rather than a murine TNF cDNA probe was used for the detection of mouse TNF mRNA. Another possibility is that we have detected mRNA for a protein that is similar but not identical to TNF. A full understanding of these data will only be possible once the 50,000 MW protein isolated by Young *et al.* (1987) is completely characterized and shown not to be TNF that is found aggregated in mast cell granules.

Mast cells and their products are involved in a variety of processes other than allergy and anaphylaxis. These include tumour cell cytotoxicity *in vitro* (Farram & Nelson, 1980; Henderson *et al.*, 1981; Ghiara *et al.*, 1985) and *in vivo* (Tanooka *et al.*, 1982; Naito *et al.*, 1984; Potter *et al.*, 1985), inflammatory joint diseases (Yoffe *et al.*, 1984; Wasserman, 1984), bone remodelling (McKenna & Frame, 1985), fibroblast growth (Atkins *et al.*, 1985; Claman, 1985) and enhancement of eosinophil toxicity in parasitic infections (Capron *et al.*, 1978; Justus & Morakote, 1981). On the other hand, recent findings indicate that TNF plays a role in the same processes. TNF has been described to be cytotoxic against tumour cells *in vitro* and *in vivo* (Carswell *et al.*, 1975; Sugarman *et al.*, 1985; Ortaldo *et al.*, 1986) and it stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts (Dayer *et al.*, 1985). TNF has also been shown to stimulate bone resorption, osteoclastogenesis (Bertolini *et al.*, 1986) and fibroblast proliferation (Sugarman *et al.*, 1985; Vilcek *et al.*, 1986). Furthermore TNF can enhance eosinophil toxicity to *Schistosoma mansoni* larvae (Silberstein & David, 1986), and there are hints that partially purified TNF can have direct cytotoxic effect on malarial parasites (Taverne *et al.*, 1984). Our data indicate that TNF can be produced by human basophil/mast cells. This seems to be also true for murine mast cells where others have detected a similar protein and where we have demonstrated the presence of TNF mRNA. The data suggest further investigations to define the exact role of basophil/mast cells in TNF-mediated processes.

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