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Synergistic effects of recombinant tumour necrosis factor and interferon-gamma on rat thyroid cell growth and Ia antigen expression

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SUMMARY

In this study, we have investigated the effects of tumour necrosis factor (TNF), interferon-gamma (IFN-γ) and interleukin-1 (IL-1) on rat thyroid cells, using the continuously growing, differentiated $FRTL_5$ cell line. No effect on cell growth was found with any cytokine alone, but the combination of TNF and IFN-γ was cytostatic. These cells were not killed in a 18-hr assay for cytotoxicity, but prolonged incubation (4 days) with TNF and IFN-γ produced cell detachment and death. Normal rat thyroid cells in primary culture were also susceptible to the combination of TNF and IFN-γ for 4 days, but not 18 hr. In addition, IFN-γ treatment induced class II (Ia) antigens on thyroid cells, and this was enhanced by TNF. TNF alone did not cause Ia expression on thyroid cells but did increase the constitutive expression of class I antigens. These results suggest that IFN-γ and TNF, which are likely to be released by the lymphocytes and macrophages infiltrating the gland in thyroid autoimmunity, may synergize to impair cell growth and produce aberrant Ia antigen expression in thyroiditis. This could be an important mechanism for disease perpetuation.

INTRODUCTION

Thyroid cell destruction and thyroid atrophy are obvious features of autoimmune thyroid disease, occurring in both human and experimental thyroiditis, but the mechanisms responsible are not fully understood. Microsomal antibodies may cause cell death by complement fixation or by antibody-dependent cell-mediated cytotoxicity (Calder *et al.*, 1973; Khoury *et al.*, 1981). In addition, T cells and natural killer (NK) cells can kill human thyroid cells *in vitro* (Sack *et al.*, 1986) and may play a role in experimental autoimmune thyroiditis (Wick *et al.*, 1982; Creemers, Rose & Kong, 1983). Another recently described feature of thyroid autoimmunity is the appearance of Ia antigens on some of the thyroid cells in the affected gland (Hanafusa *et al.*, 1983). This may play a role in the initiation or perpetuation of the autoimmune response. To date the only clearly defined modulator of thyroid cell Ia expression appears to be IFN-γ (Weetman *et al.*, 1985b), and it has become important to know whether this, or other cytokines such as TNF or IL-1, released by activated macrophages and lymphocytes, could be involved in thyroid injury more directly.

In this study we examined the effects of recombinant TNF (rTNF), IFN-γ and IL-1 on thyroid cell division, cytotoxicity and Ia antigen expression *in vitro*, using the continuously growing, differentiated thyroid cell line, $FRTL_5$ (Ambesio-Impiombato, Parks & Coons, 1980). This line was selected for thyroid-stimulating hormone (TSH) dependence and growth in low serum concentration culture medium; it is diploid and retains normal thyroid-cell functions such as iodine accumulation and cAMP production in response to TSH (Valente *et al.*, 1983; Weiss, Philp & Grollman, 1984). We also compared the results obtained using the $FRTL_5$ cell line with those of rat thyroid cell primary cultures.

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MATERIALS AND METHODS

Materials

Recombinant rat IFN-γ (purity $\geqslant 98\%$, 4×10^6 U/mg protein; Van der Meide *et al.*, 1986) was from Holland Biotechnology by (Leiden, The Netherlands); recombinant human IL-1β (purity $> 96\%$, 2×10^7 U/mg protein; Wingfield *et al.*, 1986) and TNF-α (purity $\geqslant 98\%$, $9{\cdot}6 \times 10^6$ U/mg protein) were kindly donated by Dr Alan Shaw (Biogen, Geneva, Switzerland). All contained < 100 pg endotoxin/10^6 units of cytokine (*Limulus* assay).

$FRTL_5$ cells

The mycoplasma-free $FRTL_5$ cell line came from Dr N. Marshall (University College Hospital, London). Cells were grown as described elsewhere (Valente *et al.*, 1983) in Coon's modified Ham's F-12 medium with 5% newborn calf serum (Gibco, Paisley, Renfrewshire), and six hormones (6H medium): insulin (10 μg/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 μg/ml), somatostatin (10 μg/ml), hydrocorti-

sone (10^{-8} M), all from Calbiochem, San Diego, CA, and TSH (10 mU/ml) from Armour, Kankakee, IL. Cells were passaged weekly using a collagenase/trypsin/chick serum (CTC) mixture (Ambesi-Impiombato *et al.*, 1980).

Rat thyroid cell primary cultures

Thyroids from normal Wistar and Buffalo strain rats bred in the animal house, RPMS (eight in each group) were carefully dissected and minced using a scalpel. The fragments were digested at 37° in phosphate-buffered saline containing collagenase (Sigma, St Louis, MO) 1 mg/ml and dispase (Boehringer Mannheim, Indianapolis, IN) 5 mg/ml, as described elsewhere (Weetman *et al.*, 1985b). Intact thyroid follicles were allowed to form adherent monolayers over a 2–3 day period, using the 6H culture medium described for $FRTL_5$ cells, and then removed using CTC.

Cytotoxicity

Thyroid cells ($1–5 \times 10^4$ $FRTL_5$ cells/well, 2×10^5 primary culture thyroid cells/well) were allowed to adhere for 24 hr in 96-well flat-bottomed plates (Flow, Irvine, Ayrshire). The cytokines were then added and growth inhibition measured 4 days later (unless indicated) when the wells were semi-confluent, using crystal violet staining as described elsewhere (Weetman *et al.*, 1985a). Briefly, plates were immersed in 5% formalin for 1 min to fix the viable adherent cells, washed in water and immersed in 1% crystal violet for 1 min. After further washing the dye was dissolved in 33% acetic acid and the absorbance read at 540 nm using a Titertek Multiskanner (Flow). The absorbance is thus proportional to the number of viable cells remaining in culture. An ^{111}In release assay was used to measure short-term (18 hr) cytotoxic effects. Thyroid cells, $1–5 \times 10^6$, were washed twice in RPMI-1640, resuspended in 30 μl RPMI-1640, and then incubated with ^{111}In, 15×10^6 c.p.m. in 8 μl 0·04 M HCl, and 30 μl 400 μM tropolone in saline plus HEPES 20 mM, pH 7·6. The cells were incubated for 15 min at room temperature and washed three times in RPMI-1640 with 10% fetal calf serum. We have found that this isotope labels thyroid cells much more consistently than ^{51}Cr (unpublished data). Thyroid cells, 10^4/well, were added to a 96-well round-bottomed plate together with cytokine(s) in a final volume of 150 μl and after 18 hr, 75 μl of supernatant was removed for ^{111}In counting. Spontaneously released c.p.m. (no cytokine) and total released c.p.m. (wells treated with 1% Triton X-100) were measured in each assay.

Ia expression

$FRTL_5$ cells grown in 24-well dishes were detached with CTC and stained with OX6 (anti-Ia; murine I-A homologue), OX17 (anti-Ia; murine I-E homologue) and OX18 (anti-class I antigen) monoclonal antibodies from Serotec (Bicester, Oxon), followed by goat anti-mouse Ig FITC (Coulter, Luton, Bedfordshire). The Leu 4 monoclonal antibody (Becton-Dickinson, Mountain View, CA), against human T cells and the same isotype as OX17, was used as a negative control. $FRTL_5$ cells, 10^4, were analysed for fluorescence using a Coulter EPICS C flow cytometer. Because of the limited number of rat thyroid cells from primary culture, staining was performed directly on cells adherent to multi-well glass slides. The number of cells stained and the intensity of fluorescence (graded + to + + +) was scored blind using three wells per sample.

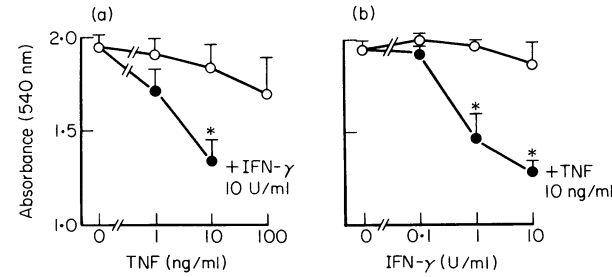

Figure 1. Cytostatic activity of TNF (a) and IFN-γ (b) alone or in combination, using $FRTL_5$ cells. In concurrent experiments with these, the absorbance of cultures treated with IL-1 did not differ from the untreated cultures; the maximum concentration of IL-1 used, 10^4 U/ml, gave an absorbance of 1·99 ± 0·11 and each addition of TNF or IFN-γ had no additional effect. Each point is the mean of six replicates; the bar is the SD. * $P < 0·001$ compared to untreated control.

Table 1. Effect of IFN-γ and TNF on ^{111}In release after 18 hr incubation

	Mean c.p.m. ± SD		
Addition to medium	$FRTL_5$ cells	Buf TFC	Wistar TFC
Nil (spontaneous release)	3406 ± 252*	1905 ± 147	9018 ± 760
1% TRITON (total release)	12447 ± 844	3796 ± 306	27810 ± 2431
IFN-γ 10 U/ml	3664 ± 318	1977 ± 91	7806 ± 592
TNF 10 ng/ml	3622 ± 289	1910 ± 157	8801 ± 1257
IFN-γ 10 U/ml + TNF 10 ng/ml	3457 ± 249	2151 ± 228	9600 ± 623

* Each figure is the mean ± SD c.p.m. of 75 μl of supernatant from 10^4 cells, six replicates each. A similar lack of effect was observed using IFN-γ 100 U/ml.

TFC, thyroid follicular cells.

Statistics

Analysis was performed using the Student's *t*-test.

RESULTS

Growth inhibition and cytotoxicity

There was no effect of IFN-γ, TNF or IL-1 alone on $FRTL_5$ cells, but there was a significant cytostatic action of TNF and IFN-γ together, as measured by crystal violet staining at 4 days (Fig. 1). No other combination of cytokines (i.e. IFN-γ plus IL-1 or TNF plus IL-1) had any effect. This action of TNF plus IFN-γ was confirmed by direct cell counts of $FRTL_5$ cells recovered from CTC-treated 24-well culture dishes after 48 hr culture. Untreated wells contained $7·2 \times 10^4$ cells per well, those with IFN-γ 100 U/ml $6·2 \times 10^4$ cells per well, those with TNF 10 ng/ml $6·2 \times 10^4$ cells/well and those with the combination of IFN-γ 100 U/ml and TNF 10 ng/ml $3·0 \times 10^4$ cells/ml. Replicate counts

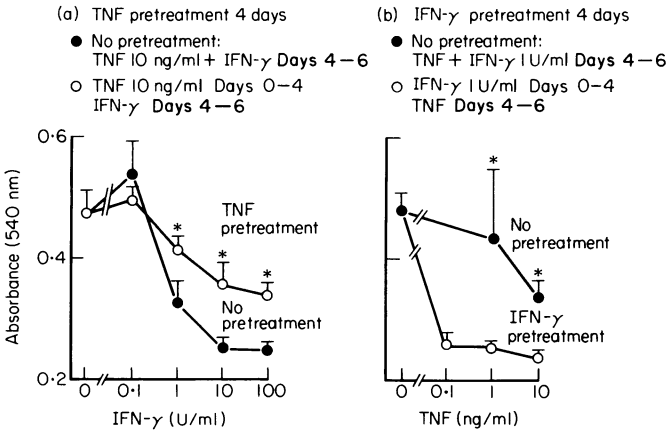

Figure 2. Effect of pretreatment with TNF or IFN-γ on crystal violet staining of $FRTL_5$ cells. $FRTL_5$ cells were pretreated (open symbols) with TNF (a) or IFN-γ (b) for 4 days; the effects of subsequent IFN-γ or TNF for 2 days are compared with TNF and IFN-γ added together for 2 days to cultures which were not pretreated (solid symbols). Each point is the mean of six replicates; the bar is the SD. * $P<0{\cdot}01$ compared to pretreated cultures.

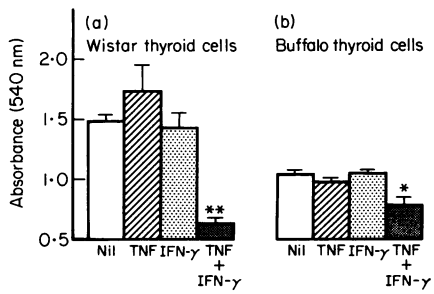

Figure 3. Effect of TNF (100 ng/ml) or IFN-γ (100 U/ml), alone or in combination, on crystal violet staining of Wistar (a) and Buffalo (b) strain thyroid cells in primary culture for 4 days. Each point is the mean of six replicates; the bar is the SD. * $P<0{\cdot}01$, ** $P<0{\cdot}001$ compared to culture without treatment (Nil). The combination of IFN-γ 10 U/ml with TNF 100 ng/ml had a less significant effect on Wistar strain thyroid cells (absorbance $1{\cdot}30\pm0{\cdot}05$) but no effect on Buffalo strain thyroid cells (absorbance $1{\cdot}09\pm0{\cdot}09$).

($n=4$) were within 10% of each mean value. None of the cytokines, alone or in combination, had any effect on $FRTL_5$ cells after 18 hr, as measured by crystal violet staining (all treatments within 10% of control value, $P>0{\cdot}1$). Moreover IFN-γ plus TNF did not affect ^{111}In release after 18 hr incubation (Table 1). However, after at least 48 hr incubation with TNF 10 ng/ml plus IFN-γ 10 U/ml (but not either cytokine alone) cells were observed to detach. These cells failed to exclude trypan blue or re-attach after washing, and this cytotoxic effect was more pronounced at Day 4.

Further analysis was performed on cells treated either with IFN-γ or TNF for 4 days, before washing *in situ* and then incubating with the other cytokine for a further 2 days. The combination of TNF for 4 days followed by IFN-γ for 2 days was less effective than adding TNF and IFN-γ together for 2 days only, whereas pretreatment with IFN-γ for 4 days greatly increased the sensitivity of the cells to TNF for 2 days (Fig. 2). As little as 0·1 ng TNF/ml was cytostatic/cytotoxic after pretreatment with IFN-γ.

Next the effect of the cytokines on normal thyroid cells in primary culture was investigated. These cells were also susceptible at 4 days to the combination of IFN-γ and TNF, but not to either cytokine alone (Fig. 3) or to IL-1 (10^4 U/ml, data not shown). There was no effect on ^{111}In release at 18 hr (Table 1). Too few cells were obtained to test IL-1 in combination with the other cytokines.

Ia expression

As described elsewhere (Weetman, Green & Borysiewicz, 1987) IFN-γ was a potent stimulator of Ia expression by $FRTL_5$ cells, which fail to express Ia under basal conditions (Table 2). Neither TNF nor IL-1 induced class II antigen expression by $FRTL_5$ cells. However, TNF increased the effect of IFN-γ on both class I and class II antigen expression and also enhanced class I expression in the absence of IFN-γ. This is shown by the right shift in peak channel number in the FACS analysis (Table 2; Fig. 4). IL-1 did not enhance IFN-induced class II antigen expression.

Analysis of rat primary thyroid cell cultures confirmed that IFN-γ, but not TNF, induced class II antigens on these cells. Untreated or TNF-treated cells failed to express detectable Ia by indirect immunofluorescence; $95\pm2\%$ were OX17 positive with 100 U IFN-γ/ml (++ intensity) and $48\pm9\%$ were OX17 positive (+ intensity) after incubation with 10 U IFN-γ/ml. The addition of TNF 10 ng/ml to 10 U IFN-γ/ml made 100% of cells positive (+++ intensity); higher concentrations of TNF in combination with IFN-γ led to cell death, so that effects on Ia could not be established. For OX6, $27\pm8\%$ of cells were positive with 10 U IFN-γ/ml (+ intensity), and this increased to $54\pm10\%$ (++ intensity) in combination with TNF 10 ng/ml.

DISCUSSION

TNF (or cachectin) is produced by macrophages and has a wide array of metabolic effects on various tissues, mediated through specific cell surface receptors (reviewed in Old, 1985; Beutler & Cerami, 1986). It is known to be cytostatic or cytolytic to a wide variety of transformed cell lines, but has little effect on normal cells (Helson *et al.*, 1975; Matthews & Watkins, 1978). More recently, cytostatic/cytotoxic synergy between TNF and IFN-γ (Williamson *et al.*, 1983; Fransen *et al.*, 1986) or IL-1 (Ruggiero & Baglioni, 1987) has been demonstrated. The synergy between TNF and IFN-γ may be due to an increase in TNF receptors by IFN-γ (Tsujimoto, Yip & Vilček, 1986; Ruggiero *et al.*, 1986), whereas the enhancement produced by IL-1 could be related to the innate cytotoxic action of this molecule on certain cells (Bendtzen *et al.*, 1986).

Our data indicate that the combination of TNF and IFN-γ is cytostatic for rat thyroid cells derived either from a functional cell line ($FRTL_5$) or from primary cultures, and prolonged incubation produced cytotoxicity for $FRTL_5$ cells. Preincubation experiments showed that this synergy probably results from a primary action of IFN which sensitizes cells to the subsequent effects of TNF; incomplete removal of TNF could account for the weaker effect seen when cultures were treated first with TNF and then IFN-γ.

Table 2. Effect of cytokines on $FRTL_5$ class I and class II antigen expression*

	Control (Leu 4)		Class II (OX6)		Class II (OX17)		Class I (OX18)	
	%†	Peak‡	%	Peak	%	Peak	%	Peak
Nil	1·3	52	2·7	50	1·2	50	98·3	132
IFN-γ 1 U/ml	1·9	50	ND		3·3	50	98·7	166
IFN-γ 0·1 U/ml	1·0	54	1·4	50	1·6	51	97·7	136
TNF 10 ng/ml	4·1	50	1·6	57	2·3	51	97·4	146
IL-1 10^3 U/ml	2·0	50	1·9	50	2·1	50	ND	
IFN-γ 1 U/ml + TNF 10 ng/ml	1·7	50	ND		72·0	63	98·8	175
IFN-γ 0·1 U/ml + TNF 10 ng/ml	2·3	50	16·3	51	14·6	50	98·4	161

* Cultures were for 4 days in the presence of cytokines.

† The percentage of cells positive for staining with each monoclonal antibody, from a total of 10^4 cells analysed.

‡ The peak channel of fluorescence intensity (log scale) by flow cytometry; the more intense the staining per cell, the higher the peak channel (treatments did not affect cell size).

ND, not done.

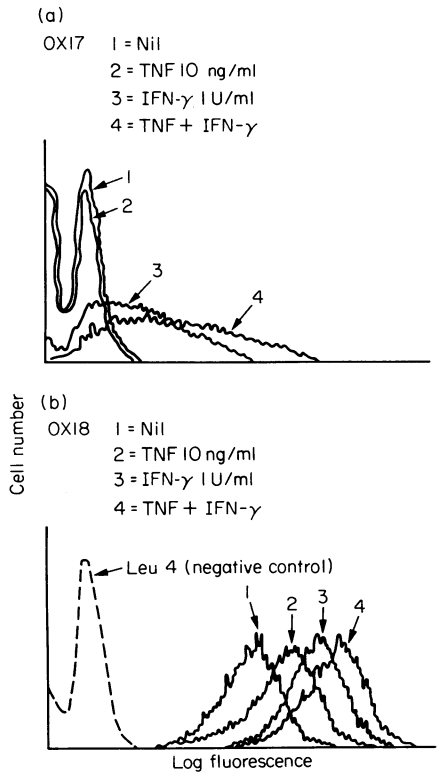

Figure 4. Effect of TNF and IFN-γ on Ia and class I antigen expression by $FRTL_5$ cells. The FACS profiles for OX17 (Ia) staining are shown in (a) and for OX18 (class I) in (b). The control monoclonal staining with Leu 4 (i.e. background staining) for both experiments is shown as the dotted line in (b); this did not vary with any of the treatments.

In contrast to experiments with islets of Langerhans' (Bendtzen *et al.*, 1986) and preliminary results on human thyroid cell primary cultures (Rasmussen *et al.*, 1986), we found no effect of IL-1 on rat thyroid cells, either alone or in combination with TNF or IFN-γ, even at high concentration. It should be noted that in these experiments we were examining effects on cell division and death, whereas the other studies determined more subtle effects of cell damage, such as loss of hormonally stimulated endocrine function. Another possibility to account for such differences may be variability, not only in tissue but also in species sensitivity to various cytokines.

We also observed that TNF enhanced IFN-γ-induced class I and class II antigen expression. TNF alone has been shown to enhance class I antigen expression on human endothelial cells and fibroblasts (Collins *et al.*, 1986) and on a murine myelomonocytic cell line (Chang & Lee, 1986). IFN-γ also enhanced Ia on this line, and synergized with TNF. Islet cells do not express Ia antigens in response to TNF or IFN alone, but do so when the two cytokines are added together (Pujol-Borrell *et al.*, 1987). Our results show that rat thyroid cells express class II antigens in response to IFN-γ but not TNF alone, with synergy between the two cytokines when added in combination.

These findings suggest that the release of TNF and IFN-γ by activated macrophages and lymphocytes infiltrating the thyroid gland in autoimmune thyroiditis could have at least two important actions, namely on thyroid cell division and destruction and on enhanced Ia expression. This latter effect may increase clonal expansion of thyroid-sensitized T cells by rendering the thyroid cell capable of presenting cell surface autoantigens. This in turn would increase the release of lymphokines, including those affecting cell migration and macrophage activation, so that a cascade of mediators and cell interactions then ensue, perpetuating the autoimmune process.

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