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Transforming growth factor-β1 inhibits activation of macrophage cell line RAW 264.7 for cell killing

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SUMMARY

Transforming growth factor β-1 (TGF-β) is a multi-potent immunoregulatory peptide that has effects on numerous cell types. Here we report that human TGF-β inhibits the activation of the macrophage cell line RAW 264.7 for killing of the L1210 tumour cell line. RAW 264.7 cells, like normal macrophages, require sequential interaction with priming and triggering stimuli for full activation of cytolytic activity. TGF-β inhibits this cytotoxicity in a dose-dependent manner at both the priming and the triggering stage. Addition of as little as 1 ng/ml TGF-β when added with either the priming signal, recombinant interferon-gamma (IFN-γ), or the triggering signal, bacterial lipopolysaccharide (LPS), completely abrogated tumouricidal activity. Incubation with TGF-β also inhibited the morphological changes normally observed in activated RAW 264.7 cells. However, TGF-β was unable to inhibit the cytotoxic activity of RAW 264.7 cells against the target cell line WEHI 164, which is sensitive to tumour necrosis factor. In contrast to the effects on cytotoxic activity, the cytostatic activity of activated RAW 264.7 cells was not inhibited by TGF-β at doses of up to 5 ng/ml. In addition, pretreatment of the L1210 target cells with TGF-β made them refractory to both the cytostatic and cytotoxic effects of RAW 264.7 cells. These data suggest that TGF-β may be an important mediator in the regulation of macrophage tumouricidal activity.

Keywords transforming growth factor-β1 macrophages

INTRODUCTION

Transforming growth factor-β (TGF-β), is a 25-kD homodimer composed of two 12·5-kD subunits held together by disulfide bonds (Sporn *et al.*, 1986). It is produced by a wide variety of cell and tissue types including platelets (Anzano *et al.*, 1982), bone (Centrella & Canalis, 1985), and soft tissues such as placenta (Frolik *et al.*, 1983) and kidneys (Roberts *et al.*, 1983). Essentially all cells have receptors for TGF-β (Roberts *et al.*, 1983). TGF-β was originally defined by its ability to cause the phenotypic transformation of rat fibroblasts (Assoian *et al.*, 1983). It has now been shown to have regulatory effects on a wide range of cell types. In the immune system, TGF-β acts as an immunosuppressive agent both *in vitro* and *in vivo*. TGF-β has been shown to inhibit T and B cell proliferation (Kehrl *et al.*, 1986b; Petit-Koskas *et al.*, 1988), macrophage maturation from haematopoietic precursors (Strassmann, Cole & Newman, 1988), natural killer (NK) and lymphokine-activated killer (LAK) cell activity (Rook *et al.*, 1986; Espevik *et al.*, 1987; Grimm *et al.*, 1988; Mule *et al.*, 1988), immunoglobulin secretion (Kehrl *et al.*, 1986a), and cytokine production (Espevik *et al.*, 1988).

It has been well established in studies performed with murine-elicited peritoneal macrophages that macrophage activation for antibody-independent tumouricidal activity is a multi-step process. Cells acquire the competence to destroy tumour cells after interactions with at least two signals (Ruco & Meltzer, 1978; Meltzer, 1981). Exposure of responsive macrophages to activated T lymphocyte products, particularly interferon-gamma (IFN-γ), results in the development of a primed cell population that can recognize and specifically bind to neoplastic cells (Marino & Adams, 1982). Treatment of the primed macrophages with a second stimulus such as bacterial lipopolysaccharide (LPS) triggers the full expression of tumour cell lysis (Weinberg, Chapman & Hibbs, 1978; Pace & Russell, 1981).

In this study we examined the effects of TGF-β on the activation of macrophages for tumour cell killing. RAW 264.7 cells, like murine elicited peritoneal macrophages, require sequential interaction with at least two signals for full activation of tumour cytolytic activity (Ruco & Meltzer, 1978; Lambert & Paulnock, 1989). Treatment of the cells with IFN-γ results in the development of a primed cell population (Pace *et al.*, 1983). Cells at this intermediate stage in the activation pathway require

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subsequent stimulation with the triggering agent LPS for full expression of tumour cell lysis (Weinberg *et al.*, 1978; Pace & Russell, 1981).

Our results indicate that TGF-β is a powerful inhibitor of both the priming and triggering stages of macrophage activation for tumour necrosis factor (TNF) independent cytotoxicity, substantially inhibiting the development of either intermediate stage in the activation pathway. In addition, the TGF-β-mediated reduction in tumouricidal activity correlated with loss of the classical 'activated macrophage' morphology normally induced by these activating stimuli. TGF-β treatment did not detectably alter the cytostatic activity of activated RAW 264.7 cells. The use of this homogeneous cell population should provide a useful system for investigating mechanisms of TGF-β-mediated regulation of the process of macrophage activation.

MATERIALS AND METHODS

Cell lines

Cell lines were maintained in RPMI 1640 tissue culture medium supplemented with 10 mM glutamine, 1 mM pyruvate, 50 U/ml penicillin and 50 U/ml streptomycin (all from GIBCO, Grand Island, NY) and 10% (v/v) of the appropriate serum. The macrophage cell line RAW 264.7 and the TNF-sensitive tumour target WEHI 164 fibrosarcoma cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in medium supplemented with fetal bovine serum (HyClone, Sterile Systems, Logan, UT). The tumour target L1210 leukaemia cell line also was obtained from ATCC and maintained in medium containing horse serum (HyClone). All incubations were performed in 7% CO_2 at 37°C in a humidified atmosphere. Cell liners were routinely monitored to ensure that they were free of mycoplasma contamination.

Cytokines

Murine recombinant IFN-γ (rIFN-γ) (specific activity 1×10^6 U/ml) was generously provided through The American Cancer Society. Highly purified human TGF-β was purchased from R & D Systems (Minneapolis, MN).

Activation of macrophages for tumour cell killing

RAW 264.7 cells were plated in triplicate in microtitre wells at effector-to-target ratios (2·5:1 and 5:1) that were previously found to be optimal (Lambert & Paulnock, 1987). After adherence for 1 h, RAW 264.7 cells were incubated for 16–18 h with murine rIFN-γ at a concentration of 20 U/ml. The medium was then removed and replaced with fresh medium containing 5 μg/ml bacterial LPS (*Escherichia coli* 026:B6, Difco Laboratories, Detroit, MI) in which the RAW 264.7 cells were further incubated for 6–8 h. When used, TGF-β was added along with the priming (IFN-γ) and triggering (LPS) agents. In some experiments transwells (Costar, Cambridge, MA) were used to separate effector from target cells.

Assay for macrophage-mediated tumour cytolysis

Macrophage-mediated tumour cytolysis was quantified by radiolabel release from L1210, or WEHI 164 where indicated, tumour target cells labelled with ^{3}H-TdR to approximately 1×10^4 ct/min per 5×10^4 cells as previously described (Lambert & Paulnock, 1987). Aliquots of the target cell suspension containing 1×10^4 labelled cells were added to the wells. Following a 72-h incubation, the amount of ^{3}H-TdR release was quantitated by liquid scintillation spectroscopy (Pace *et al.*, 1983). Total label incorporated into the target cells was assessed by solubilizing an identical aliquot of target cells with 0·5% SDS (Sigma, St Louis, MO). Spontaneous release of label into the culture supernatant was determined in all experiments using wells containing target cells alone. Results are expressed as percent specific lysis, which is calculated according to the following formula:

$$\% \text{ specific lysis} = \frac{\text{Experimental} - \text{Spontaneous release}}{\text{Total incorporated} - \text{Spontaneous release}} \times 100$$

Assessment of macrophage-mediated cytostasis

Macrophage-mediated tumour cytostasis was quantified by enumeration of L1210 tumour target cell numbers using a trypan blue dye exclusion assay. The cytostasis assay was performed as described above for cytotoxicity, except that the target cells were not labelled with ^{3}H-TdR. At various time-points during the assay, L1210 target cells in individual wells were gently resuspended with a pipet. A 0·05-ml aliquot of resuspended cells was then diluted 1:1 with a solution of trypan blue and the number of viable cells in each well counted using a haemocytometer. Triplicate wells were counted for each assay condition evaluated.

Morphometric analysis

Following activation, RAW 264.7 cells were photographed using a 35 mm Nikon camera mounted on a phase contrast Nikon inverted microscope at ×125 power. Using the photographic negatives, cell measurements were made on a digitizing tablet interfaced with a microcomputer with Bioquat System IV software (R and M Biometrics, Nashville, TN). The results are expressed as the mean ± s.e.m. of an arbitrary unit of cell circumference.

RESULTS

TGF-β inhibits priming and triggering of tumour cytolytic activity in RAW 264.7 cells

Addition of TGF-β to RAW 264.7 cells during either the priming or triggering stage of activation resulted in a dose-dependent inhibition of cytotoxicity against L1210 target cells. As can be seen in Fig. 1, the maximal inhibitory effect in both cases was consistently achieved with 1 ng/ml TGF-β. Therefore, this concentration was used in all subsequent experiments.

We also examined the kinetics of radiolabel release by target cells in the presence of TGF-β-treated or control RAW 264.7 cells (Fig. 2). Release of ^{3}H-TdR from labelled L1210 cells was not detected within the first 24 h of the cytolytic assay. However, up to 30% specific lysis of L1210 cells occurred between 24 and 72 h of co-culture with RAW 264.7 cells. Treatment of RAW 264.7 cells with TGF-β during either the priming or triggering stage resulted in a reduced level of radiolabel release between 24 and 72 h. These data suggest that TGF-β inhibited the magnitude rather than the kinetics of macrophage cytotoxicity.

Treatment of RAW 264.7 cells with TGF-β also inhibits activation-related morphological changes

We examined the effects of TGF-β on the gross morphological differences associated with activation RAW 264.7 cells. Figure 3

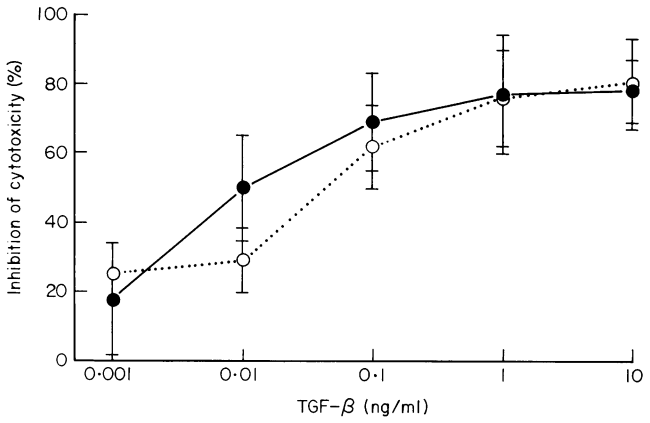

Fig. 1. Dose–response analysis of transforming growth-factor-beta (TGF-β) inhibition of activated RAW 264.7 cell line tumour cytotoxicity. Cells were incubated with TGF-β either during the priming (○) or the triggering (●) stage of activation. Results are expressed as per cent of the cytolysis by RAW 264.7 cells activated in the absence of TGF-β (per cent specific release of ^{51}Cr ranged from 17–43% in these assays). Results were calculated as the mean of five separate experiments at an effector-to-target ratio of 2·5:1.

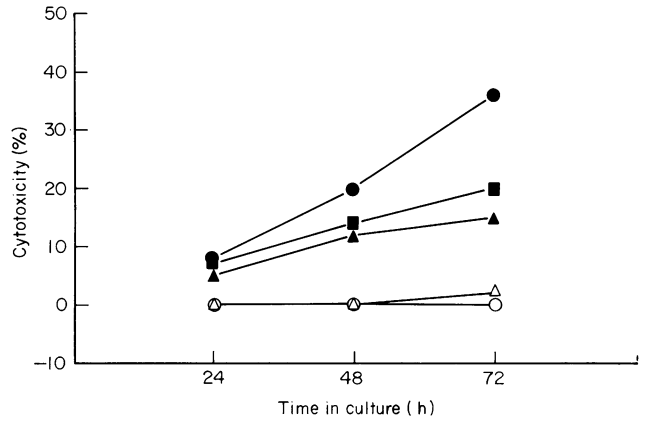

Fig. 2. Transforming growth factor-beta (TGF-β) does not alter the time course of macrophage tumour cytolysis. Cytolytic activity by activated RAW 264.7 cells (●) was evaluated at 24, 48, and 72 h after target cell addition. Parallel cultures of RAW 264.7 cells were treated with media alone (○), TGF-β alone (△), TGF-β during the priming (▲) or TGF-β during the triggering stage (■) of activation. The RAW 264.7 cells were then co-cultured with L1210 target cells and cytolysis was evaluated at 24, 48, and 72 h. Results are expressed as the mean cytotoxicity of three wells per treatment group at each time point (s.e.m. $\leq 3\%$ for each data point).

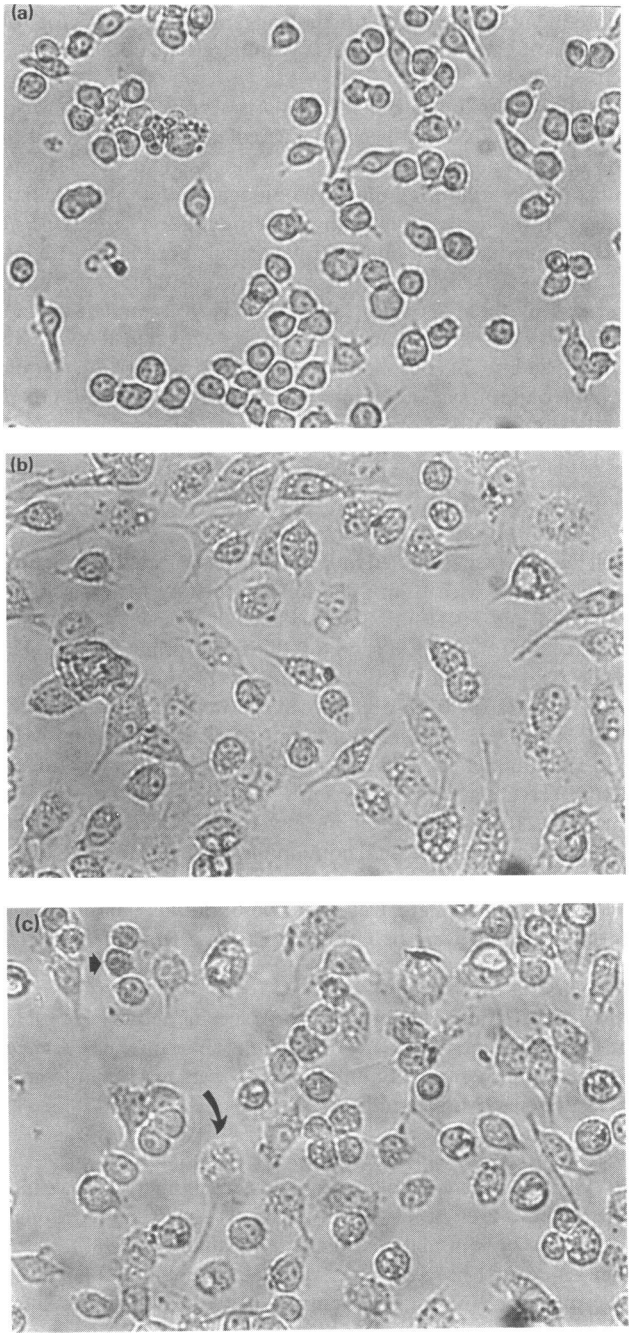

Fig. 3. Effects of activation and transforming growth factor-beta (TGF-β) treatment on the morphology of RAW 264.7 cells. Unactivated cells (a) remained small and round. After being primed and triggered with interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) effector cells (b) assumed the classical spread and highly vacuolated morphology of activated macrophages. Effector cells that were primed and triggered while exposed to 1 ng/ml TGF-β during the priming stage (c) appeared to have a bi-modal distribution of morphological phenotypes. Some cells possessed the activated morphology (arrow) while others were small, rounded and lacked vacuoles (arrow head). Magnification ×65.

illustrates that unactivated (medium control) RAW 264.7 cells were small, round, and contained no visible vacuoles (Fig. 3a). Following activation (Fig. 3b), nearly all the RAW 264.7 cells assumed the classical morphology of activated macrophages. They were flattened and spread considerably, possessed many cytoplasmic processes, and were highly vacuolated. In contrast, RAW 264.7 cells treated with TGF-β during the priming stage of activation (Fig. 3c) appeared to have a bi-modal distribution of morphological phenotypes. Some cells possessed the activated morphology (arrow) while others appeared rounded and lacked vacuoles (arrow head). Similar results were observed when RAW 264.7 were treated with TGF-β during the triggering phase of activation (data not shown). These observations

Table 1. Comparison of cell perimeters and tumouricidal activity

Treatment*					
IFN-γ	Priming TGF-β	Triggering LPS	Triggering TGF-β	Cell perimeters	Cytotoxicity (%)‡
—	—	—	—	1·96±0·09	0
+	—	+	—	5·42±0·32	25
+	—	—	—	2·39±0·16	0
—	—	+	—	2·76±0·21	2
—	+	—	+	2·51±0·13	0
+	+	+	—	4·44±0·28	5
+	—	+	+	4·36±0·31	9

* RAW 264.7 cells were cultured at 37°C for 16–18 h with 20 U/ml murine IFN-γ (priming), followed by 6–8 h with 5 μg/ml LPS (triggering) in the presence or absence of 1 ng/ml TGF-β.

† At the end of the activation procedure, photographs were taken of individual wells for subsequent computer-based morphometric analysis. Results are expressed as the mean perimeter ±s.e.m. of 50 cells/treatment group.

‡ Following activation, radiolabelled L1210 target cells were added and cytolytic activity in the cultures assessed 72 h later. Per cent specific cytotoxicity was determined as described in Materials and Methods.

IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; LPS, lipopolysaccharide.

Table 2. Comparison of the effect of TGF-β treatment on RAW 264.7 cell killing of TNF-sensitive (WEHI 164) and TNF-resistant (L1210) target cells*

	Cytotoxicity (%)†	
Effector cell treatment	L1210	WEHI 164
Medium	0	0
IFN-γ+LPS	24	50
IFN-γ+LPS+10 ng TGF-β	0	48
IFN-γ+LPS+1 ng TGF-β	0	45
IFN-γ+LPS+0·1 ng TGF-β	11	46
IFN-γ+LPS+0·01 ng TGF-β	12	50

* RAW 264.7 cells were cultured at 37°C for 16–18 h with 20 U/ml murine IFN-γ (priming), followed by 6–8 h with 5 μg/ml LPS (triggering) in the presence or absence of 0·01–10 ng/ml TGF-β.

† Following activation, radiolabelled L1210 or WEHI 164 target cells were added and cytolytic activity in the cultures assessed 72 h later. Per cent specific cytotoxicity was determined as described in Materials and Methods.

TGF-β, transforming growth factor-beta; TNF, tumour necrosis factor; IFN-γ, interferon-gamma; LPS, lipopolysaccharide.

were quantified by morphometric analysis using the Bioquat System IV software package. Cell perimeters were measured and quantified in terms of an arbitrary unit. The average surface area of activated RAW 264.7 cells was approximately twice that of unactivated cells (Table 1). Cells inhibited by TGF-β during either the priming or the triggering stage had an intermediate surface area. The corresponding cytotoxic activities of these treatment groups demonstrate that the greater surface area of activated RAW 264.7 cells correlated with increased cytotoxic activity.

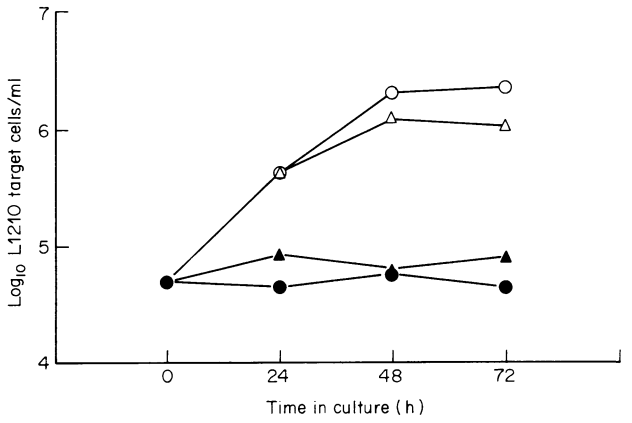

Fig. 4. Transforming growth factor-beta TGF-β does not inhibit macrophage tumor cytostasis. Target cells were incubated with medium-treated RAW 264.7 cells (○) or cells stimulated with 1 ng/ml TGF-β alone (△), activated with interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) (●), or activated with IFN-γ and LPS in the presence of 1 ng/ml TGF-β (▲). Aliquots from parallel plates were taken at each time-point for assessment of target cell number and viability by trypan blue dye exclusion. Results are expressed as the mean number of viable cells in triplicate wells.

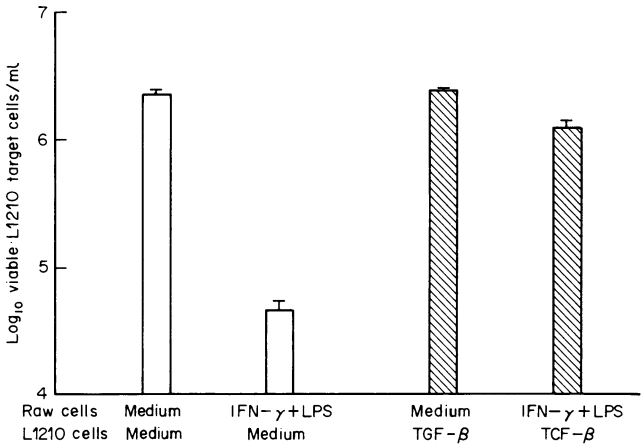

Fig. 5. Effect of transforming growth factor-beta (TGF-β) pretreatment of L1210 tumour target cells. L1210 cells were pretreated for 4 h in medium alone (open bars) or in medium containing 5 ng/ml TGF-β (hatched bars). After washing, the L1210 cells were added to cultures of control (medium) or activated RAW 264.7 cells. Viable target cell number was assessed by trypan blue dye exclusion after 72 h of culture. Results are expressed as the mean±s.e.m. of triplicate wells. IFN-γ, interferon-gamma; LPS, lipopolysaccharide.

Treatment of RAW 264.7 cells with TGF-β inhibits killing of TNF-resistant, but not TNF-sensitive target cells

We examined the ability of TGF-β to modulate the cytotoxic activity of RAW 264.7 cells on both TNF-sensitive and TNF-

resistant target cells. WEHI 164 cells have been shown to be extremely sensitive to the cytotoxic effects of TNF (Ziegler-Heitbrock & Riethmuller 1984). By contrast, we have found that L1210 target cells are resistant to the effects of both soluble and membrane bound TNF. There was no killing observed when L1210 target cells were exposed to doses of up to 1 μg/ml of recombinant TNF (data not shown). When activated RAW 264.7 cells were fixed with 1% paraformaldehyde, to assess the ability of membrane-bound TNF to kill L1210 target cells, no significant level of killing was detected (data not shown). When neutralizing antibody to TNF was added to cytotoxicity assays, killing of L1210 target cells was unaffected (32% cytotoxicity in control wells compared with 30% killing in the presence of specific polyclonal antibody to TNF). When the TNF antibody was added to cytotoxicity assays containing WEHI 164 target cells, killing was completely abrogated (42% cytotoxicity in control wells compared with 1% in wells with antibody).

Given the differences in TNF sensitivity of these two target cell lines, we examined the ability of TGF-β to inhibit selectively RAW 264.7 cell killing of the TNF-resistant L1210 cells and TNF-sensitivie WEHI 164 cells. As illustrated in Table 2, TGF-β treatment inhibited killing of L1210 cells by RAW 264.7 cells in a dose-related manner. However, RAW 264.7 cell killing of WEHI 164 cells was unaffected by TGF-β treatment of effector cells. In experiments using transwell plates, which prevent contact between effector and target cell populations, activated RAW 264.7 cells killed L1210 cells as efficiently as control wells, suggesting these target cells are killed by a soluble mediator, but one presumably distinct from TNF (data not shown).

TGF-β has no detectable effect on RAW 264.7 cytostatic activity

We also examined the effects of TGF-β on the cytostatic activity of RAW 264.7 cells, by quantification of L1210 tumour target cell numbers using a trypan blue dye exclusion assay. L1210 target cells co-cultured with unactivated (medium-treated) RAW 264.7 cells proliferated logarithmically during the first 48 h of culture (Fig. 4). In contrast, fully activated cells substantially inhibited the growth of co-cultured L1210 cells; however, TGF-β treatment of activated RAW 264.7 cells had no effect on their ability to restrict the proliferation of the L1210 target cells. These data indicate that TGF-β treatment has differential effects on the development of cytotoxic and cytostatic activities in activated RAW 264.7 cells.

Pretreatment of L1210 target cells with TGF-β makes them refractory to the anti-tumour effects of activated RAW 264.7 cells

We also examined the effect of TGF-β treatment on the susceptibility of L1210 target cells to the anti-tumour activity of activated RAW 264.7 cells. L1210 target cells were pretreated with TGF-β prior to exposure to activated RAW 264.7 cells. Washed target cells were then co-cultured with effector cells for 72 h, as described for the cytotoxicity assay. At 72 h the number of target cells were examined microscopically and counted with a haemocytometer. Viability was determined by trypan blue exclusion.

Figure 5 indicates that activated RAW 264.7 cells were able to exert their cytostatic and cytotoxic effects on untreated L1210 targets; however, TGF-β pretreated target cells proliferated nearly as well in the presence of activated effector cells as they did in medium alone. These quantitative results were confirmed by visual inspection of cultures containing activated macrophages and TGF-β-pretreated target cells (data not shown). These data suggest that L1210 target cells provide an additional signal for cytolysis which can be modulated by TGF-β pretreatment.

DISCUSSION

The data presented in this study suggest that TGF-β can play a role in the regulation of macrophage activation. TGF-β inhibited activation of RAW 264.7 cells to kill L1210 TNF-resistant target cells in a dose-dependent manner when present during either the priming or the triggering stage of activation. The loss of the classical 'activated macrophage morphology' by effector cells visually (Fig. 3) and quantitatively (Table 1) correlated with TGF-β-mediated reduction in tumouricidal activity against L1210 target cells. Interestingly, TGF-β was unable to inhibit the cytotoxic activity of RAW 264.7 cells against the TNF-sensitive target cell line WEHI 164. In addition, we were unable to detect differences in levels of TNF present in supernatant preparations from RAW 264.7 cells activated in the presence or absence of TGF-β (unpublished observations). Taken together, these data demonstrate that RAW 264.7 cells possess multiple mechanisms for target cell destruction. More important in regards to the aim of this study, TGF-β appears to spare TNF-mediated macrophage cytotoxicity (demonstrated using WEHI 164 tumour target cells) while selectively down-modulating TNF-independent cytoxicity (L1210 cells as targets). We also were unable to detect any reduction in the cytostatic activity of activated RAW 264.7 cells treated with TGF-β as compared with control-activated cells, thus suggesting that TGF-β differentially modulated the cytotoxic and cytostatic activities of RAW 264.7 cells. Finally, the tumour target cells themselves were also influenced by TGF-β, as pretreatment of L1210 cells prior to co-incubation with activated RAW 264.7 cells made them resistant to cytolysis. These data suggest TGF-β also may modulate the activity of the tumour target cells, previously proposed to provide additional signals for completion of the cytolytic activation pathway (Chapes, 1989).

At present, the mechanism(s) of TGF-β inhibition of RAW 264.7 cytotoxicity against L1210 target cells is unknown; however, TGF-β does not inhibit release of TNF from RAW 264.7 cells. TGF-β may interfere with the L-arginine-dependent cytotoxic effector mechanism of activated macrophages (Drapier, Weitzerbin & Hibbs, 1988), perhaps by blocking nitrate or nitrite synthesis. Another possibility is that TGF-β modulates receptors requisite for progression of RAW 264.7 cells through the activation pathway to cytotoxic competence. It has been previously reported that TGF-β regulates the expression of epidermal growth factor receptors in normal rat kidney fibroblasts (Assoian *et al.*, 1984) and endothelial cells (Takehara, LeRoy & Grotendorst, 1987), as well as IL-2 receptors on T cells (Kehrl *et al.*, 1986b). Alternatively, TGF-β may directly inhibit tumouricidal activity by modulating the production of key cytokines required for this activity. In this vein, it has been demonstrated that TGF-β treatment can inhibit TNF production by murine peritoneal-derived macrophages (Espevik *et al.*, 1988). Although killing of the L1210 target cells is not TNF-dependent in this system, TGF-β may inhibit the production of additional cytolytic factors produced by RAW 264.7 cells. In

this regard, it is of interest that other investigators have reported preliminary data that suggest that TGF-β has only a modest inhibitory effect on activation of peritoneal exudate macrophage TNF-dependent tumouricidal activity against fibrosarcoma 1023 cells (Nelson, Ralph & Nacy, 1989). This, combined with our findings, suggests that TGF-β has differential inhibitory effects on the generation of macrophage cytolytic activity against various tumour targets. Regardless of the mechanism of inhibition, the clear inhibitory effects of TGF-β on macrophage activation *in vitro* suggest that TGF-β may play a role in the regulation of macrophage tumouricidal activity.

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