INHIBITION OF CYTOTOXIC T CELL DEVELOPMENT BY TRANSFORMING GROWTH FACTOR β AND REVERSAL BY RECOMBINANT TUMOR NECROSIS FACTOR α

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Transforming growth factor β (TGF-β),[1] although first defined for its ability to induce nonneoplastic cells to express a transformed phenotype, has now been shown to exert multiple actions on both normal and transformed cells (1). Recent studies have also defined a variety of immunoregulatory properties of TGF-β, including inhibition of T and B cell proliferation, IL-2-R induction, IL-1-induced thymocyte proliferation, cytokine production, including IFN-γ and TNF-α, natural killer cell activity, and class II antigen expression (2–6). The mechanism(s) through which TGF-β exerts these immunoregulatory effects is at present not known.

TNF-α (also referred to as cachectin) has also been shown to express multi-functional immunomodulatory activities besides its direct cytotoxic/cytostatic effects on transformed cells (7–10). However, in contrast, to TGF-β, TNF-α enhances IL-2-R expression, class II antigen expression, and IFN-γ production by activated lymphocytes (9, 10).

The contrasting immunoregulatory activities of these two proteins prompted studies to further define their immunoregulatory activities in vitro. In this report, we describe the dose-dependent inhibition of CTL generation by TGF-β and the reversal of this inhibition by recombinant murine TNF-α (rMuTNF-α). In addition, we demonstrate that TNF-α is an important cytokine involved in CTL development.

Materials and Methods

Animals. 6–12-wk-old female BALB/c and C57BL/6 (B6) mice were obtained from Charles River Breeding Laboratories (Wilmington, MA).

Reagents. Porcine platelet–derived TGF-β (R and D Systems, Minneapolis, MN) was reconstituted in 4 mM HCl to 1 μg/ml and stored at 4°C. rMuTNF-α (sp act 7×10^7 U/mg), as determined by a standard cytotoxic bioassay using L-M cells, contained <0.025 pg of endotoxin per microgram protein by the limulus amoebocyte assay (11, 12). The specific activities of recombinant human TNF-α (rHuTNF-α) and -β provided by Genentech, Inc., as determined by the L-M bioassay were 5×10^7 and 2×10^8 U/mg protein, respectively (13, 14).

[1] *Abbreviations used in this paper:* CMEM, complete minimal essential medium; NRS, normal rabbit serum; TGF-β, transforming growth factor β.

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Mixed Lymphocyte Cultures (MLC). CTLs were generated in 5-d MLC by incubation in 24-well tissue culture plates (3524; Costar, Cambridge, MA) of 5×10^6 or 5×10^5 B6 responding spleen cells and 5×10^6 BALB/c stimulator spleen cells (irradiated 2,000 rad) per well in 2 ml of complete minimal essential medium (CMEM) consisting of Eagle's minimum essential medium supplemented with 0.1 mM nonessential amino acids, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco, Grand Island, NY) and 10% heat-inactivated FCS (Hyclone Laboratories, Inc., Logan, UT).

CTL Assay. After 5 d of culture, the cells were harvested, washed three times in CMEM and tested for cytotoxic activity in a 4-h ^{51}Cr-release assay. P815 (DBA/2 mastocytoma, H-2^d; American Type Culture Collection, Rockville, MD) or LBRM-33-1A5B (B10.BR lymphoma, H-2^k; A. Zlotnik, DNAX Research Institute for Molecular and Cellular Biology, Palo Alto, CA) were labeled with 150 μCi $Na^{51}CrO_4$ (5 mCi/ml; Amersham Corp., Arlington Heights, IL) for 45 min at 37°C, followed by three washes in CMEM. 100 μl of target cells (10^5 cells/ml) and 100 μl of effector cells at various concentrations were added in triplicate in 96-well round-bottom microtiter plates (Costar). After 4 h of incubation at 37°C and 5% CO_2 in air, the supernatants were harvested (Skatron, Rockville, MD) and their radioactivity was determined in an automatic gamma counter (Micromedic Systems, Horsham, PA). Percent specific cytotoxicity was calculated as 100 × [cpm of test supernatants of effector cells and target cells incubated together (experimental release)] − [cpm of supernatants of target cells incubated alone (spontaneous release)]/{[cpm after lysis of target cells with 2% NP-40 (maximum release)] − [spontaneous release]}. Results given are the mean of triplicate cultures ± SE. Spontaneous release of target cells alone was <10% of maximum for all experiments.

Detection of MuTNF-α in Supernatants of the MLC. The quantity of MuTNF-α produced in the MLC was determined using the WEHI-164 clone 13, which is capable of detecting levels as low as 7×10^{-4} U/ml (0.01 pg/ml) of MuTNF-α per milliliter (15). Using WEHI-164 clone 13 as indicator cells, the cytotoxicity induced by MuTNF-α was determined by an MTT tetrazolium colorimetric assay as detailed previously (15). The amount of MuTNF-α in test supernatants was calculated on the basis of cytotoxicity obtained in the presence of various dilutions of a rMuTNF-α standard. Data are expressed as units of TNF-α per milliliter.

The specificity of this assay for MuTNF-α was demonstrated by inhibiting the cytolytic activity of test supernatants with anti-rMuTNF-α antiserum produced in New Zealand rabbits. This antiserum developed by methods similar to those described for production of anti-rHuTNF-α antiserum (16), had a neutralization titer of ~10^6 U/ml in the L-M bioassay (12). Normal rabbit serum (NRS) was obtained from control New Zealand rabbits.

Results

Inhibition of CTL Generation by TGF-β. When added to MLC, TGF-β inhibited the generation of B6-anti-H-2^d–specific CTL in a dose-dependent fashion (Table I). The inhibitory effects were most pronounced when TGF-β was added during the first 24 h of the MLC (Fig. 1). Less inhibition was observed when the addition was delayed for 48 h, and only a minimal effect was seen if the delay was more than 72 h. Similarly, at the doses tested TGF-β showed no inhibitory activity if included directly in the CTL assay. These data indicate that TGF-β inhibits CTL generation in a dose-dependent manner, and its mechanism of action appears to involve early stages of the MLC.

Inhibition of TNF-α Production by TGF-β during MLC. Although a variety of in vitro immunoregulatory activities have been ascribed to TNF-α, studies examining the production of TNF-α during an MLC and the effects of altering endogenous TNF-α levels during CTL development have not been reported (8–10). Moreover, our previous studies have shown that TGF-β can inhibit TNF-α production by murine macrophages (6). Therefore, we considered it important

TABLE I
Effect of TGF-β on In Vitro CTL Generation

TGF-β* (ng/ml)	Percent specific ^{51}Cr release (± SE) at‡ E/T ratios of:			
	50:1	25:1	12.5:1	6.25:1
None	89 ± 2.0	89 ± 4.0	86 ± 0.4	75 ± 1.0
10	18 ± 2.0	8 ± 1.0	7 ± 2.0	2 ± 0.5
1.0	30 ± 0.4	16 ± 2.0	8 ± 1.0	3 ± 1.0
0.10	82 ± 0.4	79 ± 2.0	82 ± 0.2	63 ± 1.0

* TGF-β was added at the start of culture.
‡ P815 (H-2^d) target cells were used. Data are from one of three representative experiments. Percent lysis against ^{51}Cr-labeled LBRM-33-1A5B was <10% at 50:1 E/T ratio.

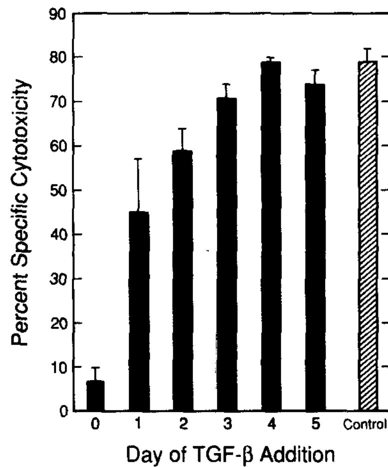

FIGURE 1. Kinetics of TGF-β inhibition of CTL generation. 10 ng/ml TGF-β were added to MLC on days shown. On day 5, this same concentration of TGF-β was added to the CTL assay. Data presented are the mean ± SE of four independent experiments performed at an E/T ratio of 25:1. Similar results were obtained at all E/T ratios tested (data not shown).

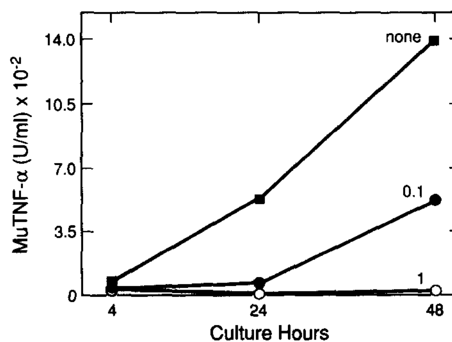

FIGURE 2. Inhibition of TNF-α production during MLC by TGF-β. 300 μl were removed from replicate MLC at the times indicated, and assayed for TNF-α as described in Materials and Methods. MLC contained no TGF-β (■), 0.1 ng TGF-β/ml (●), or 1.0 ng TGF-β/ml (○). The presence of 10 μg TGF-β in the WEHI-164 MuTNF-α bioassay did not affect the measurement of MuTNF-α activity, while the presence of anti-rMuTNF-α antiserum reduced the detection of MuTNF-α to background levels. Standard errors for all determinations were <10%. Results are presented as mean of triplicate determinations of one of four representative experiments. No significant amounts of MuTNF-α were produced by cultures containing B6 cells or irradiated BALB/c cells alone.

to examine whether the inhibitory effects of TGF-β on CTL development could in part be due to the inhibition of TNF-α production. The concentration of MuTNF-α was measured at various times during the first 48 h of MLC in the absence or presence of 1.0 or 0.1 ng/ml TGF-β. The data (Fig. 2) indicate that as early as 4 h after culture initiation, ~7 × 10^{-3} U/ml (0.1 pg/ml) of TNF-α

TABLE II

Reversal of TGF-β Inhibition of CTL Generation by TNF-α

Treatment*	Percent specific ^{51}Cr release (± SE) at‡ E/T ratios of:		
	25:1	12.5:1	6.25:1
Control	83 ± 5.0	80 ± 4.0	69 ± 4.0
10 ng/ml TGF-β	16 ± 1.0	12 ± 3.0	7 ± 2.0
10^4 U/ml rMuTNF-α	83 ± 2.0	85 ± 4.0	78 ± 1.0
10 ng/ml TGF-β + rMuTNF-α at			
10^5 U/ml	54 ± 7.0	35 ± 1.0	21 ± 0.6
10^4 U/ml	62 ± 3.0	48 ± 1.0	25 ± 2.0
10^3 U/ml	49 ± 3.0	40 ± 3.0	18 ± 1.0
10^2 U/ml	28 ± 1.0	20 ± 3.0	11 ± 2.0
10^1 U/ml	16 ± 2.0	8 ± 0.4	5 ± 1.0

* TGF-β and/or rMuTNF-α were added to MLC on day 0 at concentrations indicated.

‡ Data are from one of three representative experiments.

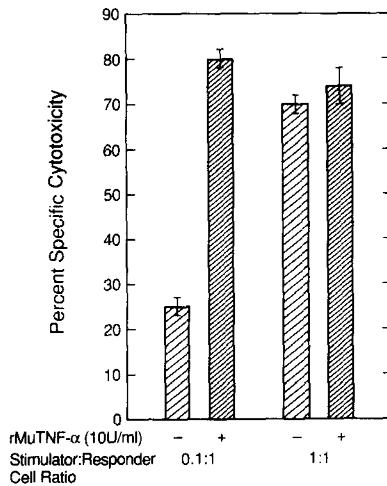

FIGURE 3. Effects of rMuTNF-α on CTL generation during suboptimal culture conditions. MLC were established at stimulator/responder ratios of 1:1 (□) and 0.1:1 (□). 10 U/ml rMuTNF-α was added on day 0 to MLC at a 0.1:1 ratio (▨) and at a 1:1 ratio (■). Results are mean ± SE of triplicate determinations performed at an E/T cell ratio of 12.5:1. Similar results were obtained at all E/T cell ratios tested (data not shown).

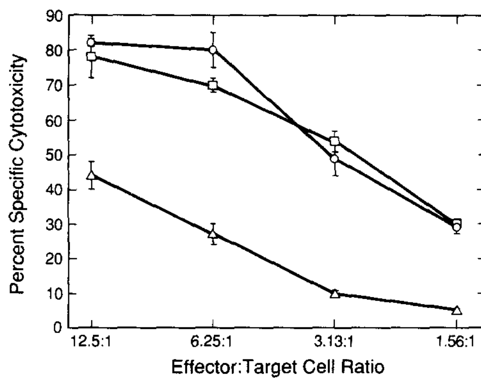

FIGURE 4. Inhibition of CTL generation by antibodies to rMuTNF-α. Rabbit serum polyclonal antibodies to rMuTNF-α was added at a 1:100 final dilution on day 0 of MLC. MLC contained: no antibodies (□); polyclonal antibodies to rMuTNF-α (△); NRS (○). Results are mean ± SE of triplicate determinations. CTL were washed three times in CMEM to prevent carryover of antibodies to rMuTNF-α into the ^{51}Cr assay.

can be detected and that TGF-β suppresses the production of TNF-α in a dose-dependent manner.

To determine if the suppression of MuTNF-α production was critical to the inhibitory activity of TGF-β on CTL generation, exogenous rMuTNF-α was added to MLC in the absence and presence of TGF-β (Table II). The addition of 10^2 U/ml of rMuTNF-α to TGF-β-suppressed MLC significantly restored the CTL activity. These results are not due to any direct activity of rMuTNF-α on the P815 target cells since the viability of these cells was not affected by rMuTNF-α doses as high as 10^5 U/ml. When rHuTNF-α was substituted for rMuTNF-α, reversal of TGF-β suppression was detected only at concentrations of 10^5 U/ml rHuTNF-α, and no activity was observed when rHuTNF-β was used at this same concentration (data not shown). These results may indicate a species preference with regard to action of TNF-α in this context, as suggested in previous studies (11).

Enhancement of CTL Development by rMuTNF-α. Under the optimal conditions used in developing the CTLs, the addition of rMuTNF-α at the doses tested had only minimal enhancing activity. However, at a suboptimal responder/stimulator ratio (0.1:1), rMuTNF-α significantly enhanced both the proliferative response (data not shown) and CTL generation to H-2^d targets (Fig. 3).

Inhibition of CTL Generation by Antibodies to rMuTNF-α. As our earlier studies demonstrated that rMuTNF-α can significantly enhance CTL development if added to MLC established at suboptimal stimulator to responder ratios, we considered it important to investigate whether antibodies to rMuTNF-α could inhibit CTL generation. As shown in Fig. 4, the addition of rabbit polyclonal antibodies to rMuTNF-α, but not addition of NRS to MLC on day 0 significantly inhibited CTL generation, further supporting our data of the critical role of TNF-α during CTL development.

Discussion

We have investigated the effects of TGF-β and rMuTNF-α on CTL generation and function. Our investigations have indicated that: (*a*) TGF-β will inhibit, in a dose-dependent manner, CTL generation, but only when TGF-β is added in the early stages of the MLC; (*b*) TGF-β does not inhibit the cytotoxic activity of CTL; (*c*) TNF-α production, which can be detected as early as 4 h after the initiation of an MLC, is inhibited by TGF-β; (*d*) addition of rMuTNF-α to TGF-β-inhibited MLC significantly reverses the inhibitory activity of TGF-β; and (*e*) antibodies to rMuTNF-α significantly inhibit CTL development. These results show that TGF-β does not inhibit CTL generation by nonspecific cytostatic or cytotoxic processes, since the addition of TGF-β to MLC for the final 72 h does not affect the CTL activity. Rather, TGF-β appears to inhibit a differentiation step in the early development of CTL. In addition, similar results have been obtained with rHuTGF-β (the polypeptide sequence of mature human and porcine TGF-β is identical) indicating that the effects observed with the natural porcine preparation are mediated by TGF-β alone, and not a contaminant (data not shown) (17).

Previous studies have indicated that CTL generation involves the production

of two or more lymphokines, including IL-2 (18–21). TGF-β has been shown to affect T cell activation by downregulating IL-2-R expression (2) and thereby inhibiting T cell proliferation. Scheurich et al. (10) have reported that rHuTNF-α can enhance the response to IL-2 by upregulating the expression of IL-2-R. Our data indicate that TNF-α plays an important role in the generation of CTL, and that TGF-β may inhibit the development of such cells, at least in part, by inhibiting TNF-α production. Our data also demonstrate that, while the reversal of TGF-β suppression is significant, it is not complete, regardless of the rMuTNF-α dose used. This may point to multiple effects of TGF-β on CTL generation, only some of which are reversible by TNF-α, and which require the presence of additional cytokines. In recent experiments, we have observed that IL-2, which induces TNF-α production, can also reverse TGF-β inhibition of CTL generation (our unpublished observations) (22). Thus, TGF-β may inhibit CTL generation directly by downregulating IL-2-R expression, or indirectly by preventing upregulation of this receptor by blocking TNF-α production. Studies are now under way to further investigate the role of TNF-α in CTL generation, and the suppression of that process by TGF-β.

Summary

The immunoregulatory effects of transforming growth factor β (TGF-β) and recombinant murine tumor necrosis factor α (rMuTNF-α) on CTL generation and activity were examined. The results demonstrate that TGF-β, in a dose-dependent manner, inhibited CTL generation but not CTL activity. The inhibitory effects were detected only when TGF-β was added within the first 48 h of the MLC. Little activity was seen when it was added thereafter, including the addition of TGF-β to the cytotoxicity assay. The production of TNF-α, which occurs during early phases of the MLC and which is inhibited in the presence of TGF-β, appears to have an important regulatory role, as altering the levels of TNF-α in an MLC can significantly influence CTL development. The inhibitory effects of TGF-β on the MLC can be significantly reversed by the addition of rMuTNF-α to the cultures. These results demonstrate that TGF-β can inhibit MLC and subsequent CTL generation at early stages of the reaction, and such inhibition may involve the suppression of TNF-α production.

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