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TGFβ down-regulates TLiSA1 expression and inhibits the differentiation of precursor lymphocytes into CTL and LAK cells

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SUMMARY

This study analysed the regulatory effects of transforming growth factor β (TGFβ) on the expression of a 70,000 MW cell surface activation antigen, TLiSA1, involved in the differentiation of cytotoxic T lymphocytes (CTL) and lymphokine-activated killer (LAK) cells from their precursor(s), and also examined the role of TGFβ in the generation of these functional cells. TGFβ was shown to suppress the expression of TLiSA1 and to inhibit, in a dose-dependent manner, the generation of both CTL and LAK cells when present from the beginning of mixed lymphocyte culture; the same inhibitory effect upon the development of cytotoxic effector cells was observed with a monoclonal antibody and with monospecific rabbit antibodies against the TLiSA1 protein. Antibody to TGFβ reversed the inhibitory effect of the cytokine on differentiation and on TLiSA1 expression. Exogenous IL-2 or, to a lesser extent, tumour necrosis factorα (TNFα) added to mixed lymphocyte cultures (MLC) augmented both TLiSA1 antigen expression and cytotoxic function by the resulting blast cells; the co-addition of TGFβ inhibited both of these cytokine-mediated effects. Similarly, it was shown that phytohaemagglutini (PHA)-induced lymphoblasts up-regulate their surface expression of TLiSA1 and exhibit increased LAK activity in response to IL-2, and TGFβ inhibited both of these events; this IL-2-induced increase in LAK cell function was also inhibited by antibodies to TLiSA1. It is suggested that TLiSA1 antigen expression is intimately linked to the differentiation of cytotoxic effector cells and that such differentiation may be a distinct process from IL-2-induced proliferation, although both events can be regulated by TGF-β.

INTRODUCTION

Transforming growth factorβ (TGFβ) is a member of a family of polypeptide factors that regulate growth and differentiation in a variety of cell types (reviewed by Massague, 1987). TGFβ has been purified and cloned (Derynck *et al.*, 1985), and it has been found to exert profound effects on cells of the immune system: thus the purified factor inhibits the production of interferon-gamma (IFN-γ) by peripheral blood mononuclear cells (PBMC), and the production of tumour necrosis factor (α) (TNFα) by macrophages (Esperik *et al.*, 1987); it depresses the cytolytic activity of large granular lymphocytes (NK cells) and their responsiveness to IFN-α (Rook *et al.*, 1986); and it suppresses the growth of mouse thymocytes and human B and T lymphocytes (Kehrl *et al.*, 1985, 1986; Ristow, 1986).

The mechanism by which TGFβ regulates the growth of human T cells has been investigated by Kehrl *et al.* (1986b), and it has been shown that TGFβ inhibits the IL-2-induced up-regulation of the IL-2 (Tac) and transferrin receptors. This same group also reported that TGFβ inhibited the differentiation of human B lymphocytes, and demonstrated that this effect was not due solely to its effect on B-cell proliferation (Kehrl *et al.*, 1986a). In a study of the role of TGFβ upon a development of cytotoxic T lymphocytes (CTL) by murine splenocytes in mixed lymphocyte cultures, Ranges *et al.* (1987) found that the addition of TGFβ from the beginning of culture inhibited the development of CTL and that this effect was partially reversible by the addition of TNFα, the production of which was suppressed by TGFβ. These data indicated that TGFβ inhibited CTL generation by the suppression of TNFα production.

We have reported previously that a monoclonal antibody against an activation antigen of 70,000 MW (TLiSA1) on T cells inhibited the differentiation of precursor cells into effector CTL and lymphokine-activated killer (LAK) cells (Burns *et al.*, 1985). It was postulated that the TLiSA1 antigen, so called because it was thought to be 'T-lineage specific', was a receptor

Abbreviations: CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IL-2, interleukin-2; LAK, lymphokine-activated killer; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; NK, natural killer; PBM, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; TGFβ, transforming growth factorβ; TNFα, tumour necrosis factorα; TLiSA, T-lineage-specific activation antigen.

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for a T-cell differentiation factor. Because of its possible involvement in such receptor function, it was of interest to consider the effect of treatment with TGFβ upon the differentiation of human CTL and LAK cells and to measure any influence of this factor upon TLiSA1 antigen expression.

MATERIALS AND METHODS

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of normal individuals by collecting cells from the interface after centrifugation over lymphoprep (Nygard, Oslo, Norway). The PBMC were washed and resuspended in RPMI medium containing 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, Ayrshire), 5×10^{-5}M 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml) (TC medium). For preparation of tonsil-conditioned medium (T-CM), single cell suspensions of tonsil cells were cultured in the presence of 5 μg/ml phytohaemagglutinin (PHA; HA16, Wellcome Reagents, Dartford, U.K.) for 18 h at a cell density of 1×10^7/ml in TC medium. T-CM was recovered by centrifugation, filtered (0·22 U) and stored at $-20°$. PHA-T lymphoblasts were generated by culturing PBMC in the presence of 2 μg/ml PHA for 8 days at a cell concentration of 2×10^6/ml in TC medium. On Days 3 and 5, the cultures were fed with T-CM (20–30%). Blasts were isolated on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient.

Cell lines

An allogeneic B-lymphoblast (BL) clone, N23, was established from the PBMC of a normal donor transformed with Epstein–Barr virus (EBV) (Watson, Burns & Mackay, 1983); these cells strongly expressed both Ia and CD20 (B1) antigens when analysed by FACS analysis. The same cells were used as target cells in cytotoxic assays as a measure of specific CTL function. For analysis of LAK cell-mediated cytotoxicity, cells from the human pigmented melanoma cell line, LiBr (Burns, Triglia & Werkmeister, 1984) were used. All of the cell lines were demonstrated to be free of mycoplasma, as described elsewhere (Triglia & Burns, 1983).

Antibodies

Mouse monoclonal antibodies to the CD antigens were purchased from Coulter Corporation, Sydney, NSW. The anti-Ia antibody used was FMC52 (kindly provided by Dr H. Zola, Flinders Medical Centre, Adelaide). Rabbit antibody to TGFβ was purchased from R and D Systems Inc. (Minneapolis, MN). The LeoA1 monoclonal antibody against TLiSA1 was purified by high-performance liquid chromatography (HPLC) from ascites fluid raised in BALB/c mice primed with pristane. The rabbit anti-TLiSA1 antiserum (A1) was prepared by immunizing a female NZ white rabbit with immunopurified TLiSA1 antigen immunoblotted onto nitrocellulose paper and solubilized in DMSO. The first intramuscular injections were given with complete Freund's adjuvant, and the three subsequent injections 3 weeks apart were given with incomplete Freund's adjuvant. Antibodies were purified from the serum by affinity chromatography on protein A, and $F(ab')_2$ fragments were prepared with standard pepsin treatment and analysed by SDS–PAGE. The antibodies gave a single band at 70,000 MW when analysed by immunoblotting of lysates of PHA blasts and also immunoprecipitated a single band at 70,000 MW from surface-labelled cells; the rabbit antibodies also blocked the binding of radio-labelled LeoA1 monoclonal antibody.

Factors

Human-transforming growth factor type β1 (hTGFβ1) was purchased from R and D Systems Inc. Recombinant interleukin-2 (rIL-2) (Lot LP-339) was kindly provided by Cetus Corporation (Emeryville, CA); it was 97% pure by SDS–PAGE analysis and contained 0·006 ng endotoxin per 10^6U. Recombinant human tumour necrosis factor α (TNFα) was provided by Genentech Inc., San Francisco, CA.

Induction of CTL and LAK cells

CTL were produced from mixed lymphocyte cultures of 2×10^6 PBMC from normal subjects with 1×10^5 allogeneic B lymphoblasts irradiated with 3000 rads in 4 ml of TC medium. Flasks (Falcon 3013) containing the cells were maintained upright for 9–10 days in 95% air/5% CO_2 at 37° in a water-saturated atmosphere, and were supplemented with 1 ml of additional TC medium after 5 days. The cultures that were used to induce CTL also generated high levels of LAK cells.

Cytotoxicity assay

A ^{51}Cr-release assay was employed as described elsewhere (Burns, Boyd & Beverley, 1982). Target cells were labelled with ^{51}Cr (Amersham) by mixing 1×10^6 cells with 50 μCI of ^{51}Cr at 37° for 1 hr. The labelled target cells were washed and placed (100 μl at 5×10^4/ml) into the wells of V-bottomed microtitre plates (Linbro/Titertek, Flow Laboratories Inc.). and different concentrations of effector cells were added in a volume of 100 μl. Plates were centrifuged (400 *g*) for 1 min and were incubated at 37° for 4 hr before recentrifugation, as before. One-hundred microlitres of supernatant were removed from each well, the released radioactivity was counted in a gamma counter, and the results were calculated as mean percentage specific lysis (Burns *et al.*, 1982). Significant differences between values were calculated by the one-tailed Student's *t*-test. The CTL were able to kill the stimulating B lymphoblasts. The LAK cells were tested by their ability to kill the LiBr melanoma cell line, a cell which is insensitive to lysis by natural killer (NK) cells (Burns *et al.*, 1982).

Cell staining and flow cytometric analysis

Cultured cells were stained by indirect immunofluorescence and analysed by flow cytometry (Coulter EPICS V, Coulter Electronics, Inc.).

Competitive binding assay

HPLC-purified LeoA1 antibody was labelled with^{125}I to 10 μCi/μg protein by the chloramine-T method. For the competitive binding assay, 5×10^5 cells were incubated with serial dilutions of radiolabelled LeoA1 in a total volume of 100 μl of RPMI-1640 medium containing 1% bovine serum albumin and 20 mM HEPES for 30 min at 4°. After incubation, cell-bound and free ^{125}I-LeoA1 were separated by centrifugation on a layer of 80% silicone oil (Dow Corning 550 fluid; Dow Corning Australia Pty. Ltd, N.S.W.)/20% paraffin oil. The tip of the tubes that contained the cell pellet was cut off and radioactivities were measured in a gamma counter. Non-specific binding was estimated by adding a 200-fold excess of unlabelled LeoA1

antibody in the binding assay. In some experiments, cells were pretreated with hTGFβ (2 ng/ml) or rIL-2 (200 U/ml) for 20 hr at 37° before the LeoA1 binding assay. On the ordinate was plotted B/F, which is equal to the bound counts divided by free counts. On the abscissa was plotted the molecules of antibody bound per cell.

RESULTS

Effects of TGFβ on the phenotype of cells generated in mixed lymphocyte culture: inhibition of expression of the TLiSA1 antigen

It has been reported previously that TGFβ inhibits the IL-2-dependent proliferation of T cells (Kerhl *et al.*, 1986b), hence for any assessment of the role of this factor in T-cell differentiation it was important to establish that the cells underwent activation and blast transformation in the presence of TGFβ. This criterion was met by morphological analysis of cells from the MLC when it was shown that by Days 8–10 of culture most (>90%) of the cells were blasts whether or not TGFβ was present, and this was confirmed by routinely making cytocentrifuge preparations and staining with Giemsa. As is shown in Table 1, however, there was always a considerable decrease in the numbers of T-cell blasts obtained from MLC performed in the presence of TGFβ (mean reduction, 54% in six experiments with 2 ng/ml) and this reduction in proliferation caused by TGFβ made invalid any attempt to assess the role of this factor by limiting dilution analysis.

Another important consideration in assessing the role of TGFβ on the development of CTL is perturbation in the surface marker phenotype of the resulting T-cell population. Kehrl *et al.* (1986b) already established that treatment with TGFβ did not alter the expression of CD3 on previously activated T cells; for the present study we have demonstrated that the surface expression of CD3, CD4, CD8 and CD20 remains relatively unaltered following MLC in the presence of TGFβ (Table 2). In contrast, expression of the IL-2 receptor, identified by binding of CD25 (anti-Tac) antibody, was usually but not invariably reduced by the presence of TGFβ (Table 2). Expression of the TLiSA1 antigen was invariably reduced by TGFβ during MLC (Table 2) in a dose-dependent manner (Fig. 1).

Table 1. Influence of TGFβ on T-cell proliferation during MLC

Concentration of TGFβ	Cell number/flask ($\times 10^{-6}$)*	C.p.m.†	% reduction in cell number	% reduction in C.p.m.
0	4·0, 2·1	63,459 ± 10,028	0, 0	0
0·02 ng/ml	4·0, 2·0	51,509 ± 9206	0, 4·8	18·8
0·2 ng/ml	3·6, 1·3	37,027 ± 5166	10, 38·1	41·7
2 ng/ml	2·1, 1·1	19,750 ± 4465	47·5, 47·6	68·9
5 ng/ml	—, 0·7	—	—, 66·7	—
10 ng/ml	1·7, —	22,450 ± 2954	57·5, —	64·6

*MLC were established in flasks with irradiated B lymphoblasts and 2×10^6 PBMC per flask as described in the Materials and Methods. The TGFβ was added at the beginning of culture and the viable cells counted after 8–10 days of culture. The figures shown are the viable cells counted, and the results for two independent experiments are shown.

†The c.p.m. are those obtained after a 6-hr pulse with tritiated thymidine on Day 6 of MLC established in microtitre wells. The data shown are the mean ± SD of six wells for each concentration.

Inhibition of the generation of CTL and LAK cells by TGFβ

Figure 2 illustrates one typical example of five similar experiments in which the presence of TGFβ from the beginning of MLC almost totally abrogated the development of CTL and LAK cells. This influence on the inhibition of generation of both CTL and LAK effector cells was dose related (Fig. 1), and for both cell types maximum inhibition was reached at a concentration of around 2 ng/ml, similar to the concentration that caused the maximum reduction in TLiSA1 expression. The presence of TGFβ during the cytotoxic phase of the assay had no effect and the factor had to be introduced at the beginning of the MLC for maximum effectiveness.

To confirm the specificity of the effect of TGFβ, rabbit antibodies to this factor (25 μg/ml) were added to the TGFβ 1 hr prior to addition to the cell cultures. This procedure resulted in total abolition of the inhibitory effect of TGFβ and indeed slightly augmented the cytotoxic function of the resulting effector cells—possibly due to the removal of TGFβ in the fetal calf serum—but confirming the specific role of TGFβ in this system (data not shown). The presence of this antibody also reversed the effect of TGFβ upon TLiSA1 expression and such expression was slightly elevated above control values seen in the absence of either TGFβ or antibody.

We have reported previously that a monoclonal antibody against the TLiSA1 antigen inhibited both CTL and LAK cell differentiation (Burns *et al.*, 1985); this was confirmed in the

Table 2. Regulation of surface marker phenotype of lymphocytes generated in MLC by TGFβ and IL-2

		% positive (MIF)*			
Lymphocyte markers		Nil	TGFβ	IL-2	IL-2 +TGFβ
Exp. 1	CD3	89·1 (144)	92·9 (152)		
	CD4	45·2 (73)	53·3 (84)		
	CD8	38·4 (68)	33·3 (68)		ND
	CD20	5·3 (11)	4·4 (13)		
	LeoA1	42·6 (55)	15·3 (49)		
	CD25	32·1 (42)	13·5 (31)		
Exp. 2	CD3	70·2 (102)	73·1 (99)	74 (110)	
	CD4	30·1 (39)	30·0 (37)	17 (32)	ND
	CD8	43·4 (64)	40·0 (64)	56 (93)	
	CD20	3·5 (8)	2·0 (11)	19 (33)	
	LeoA1	40·5 (44)	5·7 (20)	70 (68)	22 (36)
	CD25	8·3 (11)	9·8 (19)	47 (54)	26 (29)

Cells were generated in MLC for 9 days in the presence or absence of TGFβ (2 ng/ml) or of IL-2 (1000 U/ml) as described in the Materials and Methods. At the end of culture, the cells were stained by indirect immunofluorescence and analysed by flow cytometry.

*MIF, mean intensity of fluorescence in arbitrary units from flow cytometric analysis.

ND, Not determined.

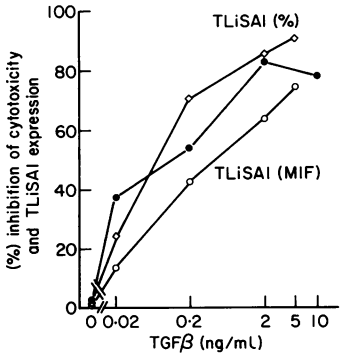

Figure 1. Concurrent inhibition of TLiSA1 antigen expression and of the development of cytotoxic effector cells against LiBr (melanoma) cells by TGFβ in MLC. TGFβ at 5, 2, 0·2 or 0·02 ng/ml or medium alone was added at the beginning of MLC. On Day 9 the resultant cells were washed and stained with LeoA1 monoclonal antibody and FITC-labelled sheep F(ab')$_2$ anti-mouse IgG and analysed by flow cytometry; cytotoxicity was assessed with the indicated target cells in a 4-hr ^{51}Cr-release assay at an effector : target ratio of 20 : 1. The results are presented as the inhibition of the percentage of cells positive for TLiSA1 (◇) and the mean intensity of fluorescence on an arbitrary scale from flow cytometry (○), and of the mean specific cytotoxicity (●).

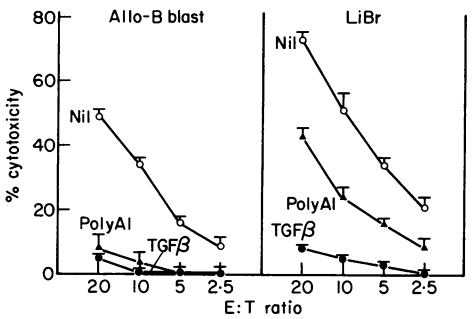

Figure 2. Effects of TGFβ and rabbit anti-A1 polyclonal antibody on CTL and LAK cell cytotoxic functions. 2 ng/ml TGFβ, rabbit anti-A1 antiserum (1 : 200), or medium alone were added at the beginning of MLC. Cytotoxic function was tested on Day 9 in a 4-hr ^{51} Cr-release assay.

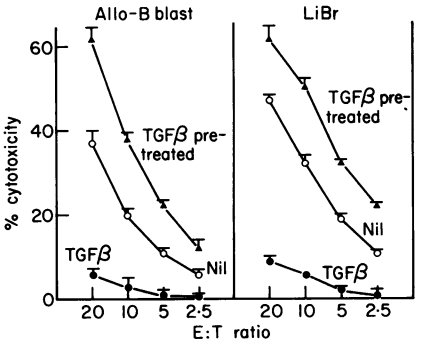

Figure 3. Effect of pretreatment of B blasts with TGFβ on CTL and LAK cytotoxic functions. B lymphoblasts were washed and resuspended in fresh medium to 2 × 10^5 cells/ml and evenly split into identical pairs of flasks. To one flask of each pair was added either 2 ng/ml TGFβ or medium alone. The cells were cultured for a further 2 days, washed and irradiated for use as stimulating cells in MLC with a single starting population of PBMC. The results labelled TGFβ received 2 ng/ml TGFβ at the beginning of mixed lymphocyte culture. On Day 9 CTL and LAK cytotoxic functions were assessed in a 4-hr ^{51}Cr-release assay.

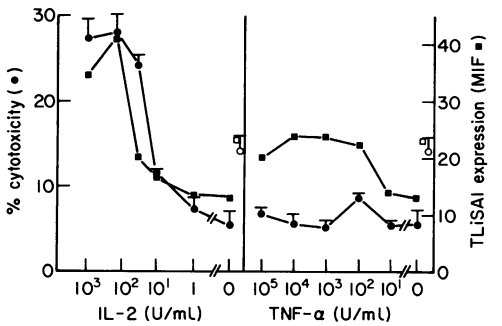

Figure 4. Reversal of the inhibitory effects of TGFβ by IL-2 and TNFα. 2 ng/ml of TGFβ plus IL-2 or TNFα were added to MLC on Day 0 at concentrations indicated. On Day 8 LAK cytotoxicity (●) was measured in a 4-hr ^{51}Cr-release assay at an effector target ratio of 2·5 : 1. The cultured cells were also stained with LeoA1 monoclonal antibody followed by FITC-labelled sheep F (ab')$_2$ anti-mouse IgG and analysed by flow cytometry; results are shown as the mean intensity of fluorescence (MIF, ■). (○) and (□) indicate the cytotoxicity and MIF of control (Nil) group, respectively.

present study with the monoclonal antibody (not shown) and with a monospecific rabbit polyclonal antibody raised against the purified TLiSA1 protein, and the inhibition was comparable with that mediated by TGFβ (Fig. 2). We also reported (Burns *et al.*, 1985) that the monoclonal antibody did not inhibit T-cell proliferation. In the present study, the presence of the monoclonal antibody at relatively high concentrations (16 μg/ml) did slightly inhibit the numbers of cells produced during MLC, however this clearly did not account for the failure of T cells to differentiate since the rabbit antibody that also inhibited differentiation (Fig. 2) slightly augmented T-cell proliferation in MLC. [Like TGFβ, the monoclonal antibody had to be present from the initial stages of MLC in order for inhibition of differentiation to occur (data not shown; Burns *et al.*, 1985).]

It was important to demonstrate that the presence of the factor was inhibiting subsequent differentiation and not simply preventing the initial activation step. This aspect was considered by examining the effect of pre-treating with TGFβ the B lymphoblasts used as stimulator cells in mixed lymphocyte cultures. B lymphoblasts used for stimulation were washed and resuspended in fresh medium to 2 × 10^5 cells/ml. The cells were evenly split into identical pairs of flasks. To one flask of each pair was added TGFβ (2 ng/ml) and the cells cultured for a further 2 days. At this time a sample was removed from each flask for testing for viability, cell count and surface Ia antigen expression. In each case it was found that the viability and cell count was unaffected by the factor, and indirect immunofluorescent staining and flow cytometric analysis showed only a small decrease in antigen expression induced by TGFβ (99% positive with MIF165 in controls versus 96% positive MIF151 in TGFβ treated). The remaining cells in each flask were then irradiated under identical conditions for use in mixed lymphocyte cultures.

The results from such mixed lymphocyte cultures showed that preincubating the B blasts with TGFβ did not prevent the

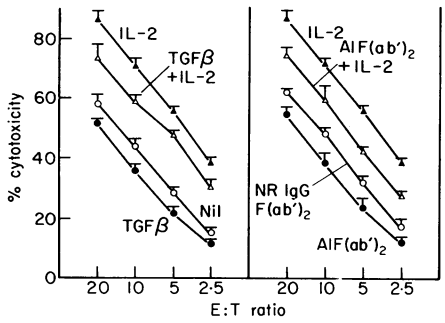

Figure 5. Reduction of IL-2-induced augmentation of LAK killing generated from PHA-stimulated T lymphoblasts by TGFβ and $F(ab')_2$ fragments of rabbit anti-A1 antibody. Cell suspensions of PBMC (2×10^6/ml) containing 2 μg/ml PHA were cultured on Day 0 with 125 U/ml IL-2, 2 ng/ml TGFβ, IL-2 plus TGFβ, medium alone, 40 μg/ml anti-A1 $F(ab')_2$, IL-2 plus anti-A1 $F(ab')_2$, or 40 μg/ml normal rabbit IgG $F(ab')_2$. On Days 3, 5 and 7, all cultures were fed with 5 U/ml IL-2. On Day 9 the Day 0 protocol was repeated together with the addition of 5 U/ml IL-2 into each culture. LAK cytotoxic function was tested on Day 12.

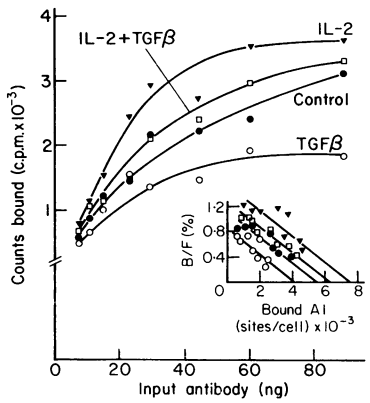

Figure 6. Regulation of A1 expression on PHA blasts by TGFβ and IL-2. PBMC suspensions at 2×10^6/ml were cultured with 2 μg/ml PHA. On Days 3 and 5, cultures were fed with T-CM. On Day 7, 200 U/ml IL-2, 2 ng/ml TGFβ, IL-2 plus TGFβ or medium alone were added to cultures for a further 20 hr. The blasts were then isolated on a discontinuous Percoll gradient. The competitive binding assay was performed as described in the Materials and Methods. In the inset the same data is plotted for Scatchard analysis. On the ordinate is plotted B/F, which is equal to the bound counts divided by free counts. On the abscissa is plotted molecules of ^{125}I-labelled LeoA1 bound per cell.

subsequent generation of CTL or LAK effector cells. Indeed, as is shown for one experiment in Fig. 3, such pretreatment of the B lymphoblasts augmented the production of these effector cells.

Reversal of the inhibitory effects of TGFβ by IL-2 and TNFα

In a murine system, Ranges *et al.* (1987) demonstrated that the addition of exogenous TNFα to their MLC partially reversed the inhibitory effect of TGFβ on the development of CTL. That such reversal also occurs in human cells with TNFα is illustrated in Fig. 4. However, the quantitative effect of TNFα was very slight, and was only significant at a single concentration (100 U/ml). This was not a toxic effect since the presence of increasing concentrations of TNFα in these cultures did not reduce the viability or the numbers of effector cells generated in these mixed lymphocyte cultures (data not shown). By contrast, in this human system, the effect of added IL-2 was much more dramatic and this factor reversed the inhibitory effect of TGFβ in a dose-dependent manner (Fig. 4). In addition, IL-2 (and TNFα) increased the relative expression of TLiSA1 (Table 2 for IL-2; and TNFα increased TLiSA1 MIF from 40 to 44), and partially reversed the inhibitory effects of TGF β upon such antigen expression (Fig. 4).

To further analyse the contrasting functional effects of the presence of IL-2 and TGFβ, PBMC were stimulated with PHA in the presence or absence of these factors from the beginning of culture then analysed for phenotype and tested for LAK cell function. By indirect immunofluorescence and analysis by flow cytometry, it was found IL-2 and TGF-β respectively, increased and reduced Tac and TLiSA1 antigen expression of the resulting blast cells, and that TGFβ prevented the increase induced by IL-2; CD3, CD4 and CD8 antigen expression remained unaltered (data not shown). In the LAK functional assay, it was found (Fig. 5) that treatment with IL-2 augmented killing whereas treatment with TGFβ reduced the generation of LAK cells ($P < 0.05$) in this system. TGFβ also caused a reduction in the IL-2-induced augmentation of killing. For comparison, similar cultures were set up with $F(ab')_2$ fragments of the rabbit A1 serum (anti-TLiSA1), and in these experiments the control cultures contained the same concentration of $F(ab')_2$ fragments of normal rabbit serum. The results (Fig. 5), although less striking than those obtained in LAK assays following MLC, were significant ($P < 0.05$), and the inhibitory effects of the A1 antibodies were exactly comparable to those induced by TGFβ.

TGFβ down-regulates TLiSA1 expression and inhibits the IL-2-mediated increase in antigen expression

From the experiments described above it was evident that IL-2 (and TNFα) not only reversed the inhibitory effects of TGFβ upon the generation of cytotoxic effector cells, but themselves caused significant augmentation of cytotoxic function. The effect of TGFβ in cultures containing exogenous IL-2 (or TNFα) was to inhibit the development of this augmented cytotoxicity (Fig. 5), an effect that could not be overcome with increased concentrations of IL-2 or TNFα. Thus it appears that TGFβ may be operating at two levels: one by inhibiting cytokine release, and two by inhibiting the responsiveness of the cells to cytokines, perhaps by modulating receptor expression.

To test the direct effect of TGFβ upon TLiSA1 expression, T lymphoblasts were generated by stimulation with PHA followed by culture for 8–10 days in conditioned medium containing IL-2. The cells were cultured for a further 2 days without the addition of CM then tested for the effect of TGFβ and or IL-2 addition for various periods of time. At this time the blast cells were harvested, purified on Percoll gradients, and examined for surface antigens by indirect immunofluorescence and flow cytometry. The results demonstrated that the addition of IL-2 for 12 hrs before testing increased the surface expression of TLiSA1 and Tac without inducing any changes in CD3 expression (data not shown). The changes to TLiSA1 antigen expression induced by treating the lymphoblasts with IL-2 or TGFβ or both for 20 hr were further examined in a radioligand binding assay (Fig. 6), and it was found that IL-2 up-regulated TLiSA1 expression whereas TGFβ down-regulated expression

of the antigen, and TGFβ also prevented the IL-2-induced up-regulation of antigen expression. The slopes of the Scatchard plots indicated that the affinity of antibody binding was not altered, rather the number of antigen molecules expressed appeared to change. The increased antigen expression induced by IL-2 required both protein and RNA, but not DNA, synthesis (data not shown).

DISCUSSION

In this report it is shown that TGFβ and polyclonal antibodies to the purified TLiSA1 antigen both prevent the formation of mature CTL and LAK cells during the course of MLC culture. Circumstantial evidence for a common mechanism of action is provided by the observation that the presence of TGFβ from the beginning of culture inhibits expression of the A1 activation antigen on the resulting T lymphoblasts.

That TGFβ was inhibiting the differentiation of these effector cell types by directly interfering with the cells themselves was shown in several ways. Thus (i) pretreatment of the stimulating B lymphoblasts did not reduce the generation of cytotoxic effector cells; rather such treatment increased the numbers and effector function of the cytotoxic cells generated. The nature of this effect must remain speculative: it may be that TGFβ increases the surface expression of Epstein–Barr viral antigens making the cells better stimulators, and certainly the functional effects of TGFβ on EBV-transformed B lymphoblasts differ from those on normal B lymphocytes (Blomhoff *et al.*, 1987). (ii) Blast transformation of PBMC in the MLC, as assessed morphologically, was not affected by TGFβ, and the LAK function of purified T lymphoblasts isolated on Percoll gradients after their production by PHA stimulation and culture in IL-2 was found to be reduced by the presence of TGFβ. (iii) Conversely, the presence of TGFβ during the cytotoxic phase of the assay had no effect on the function of either CTL or LAK cells, and this result is in keeping with the lack of effect of the factor on the effector phase of natural killer (NK) cell function (Rook *et al.*, 1986).

It has been shown that TGFβ suppresses cytokine production (Esperik *et al.*, 1987), and it could be deduced that its effect in MLC is the result of a paucity of cytokines. The relative lack of cytokines would reduce the cytokine-driven up-regulation of antigens such as TLiSA1 and Tac, and indeed the addition of cyclosporin A to cultures of PHA-induced T lymphoblasts prevents increased TLiSA1 antigen expression (B. Jin, unpublished data), an effect in keeping with its role in inhibiting cytokine production. That such a mechanism is involved in the TGFβ-mediated inhibition of the development of murine CTL was recently demonstrated by Ranges *et al.* (1987). These authors found that the presence of TGFβ in MLC of splenic cells inhibited the production of TNFα and the subsequent development of CTL, but that the addition of exogenous TNF partly restored CTL development. But experiments reported here involving the addition of exogenous IL-2 and TNFα indicated that the inhibition of cytokine production is not the only mode of action of TGFβ. In these experiments it was found that added IL-2 or, to a lesser extent, TNFα considerably augmented the cytotoxic function of effector cells generated in MLC, and that TGFβ inhibited this additional stimulatory effect; similar results were obtained with LAK cells induced by PHA rather than in MLC. Hence it appeared that TGFβ might be regulating the responsiveness of the cells to cytokines, an effect in keeping with the role of this factor in other systems where it has been shown to regulate receptor expression (Kerhl *et al.*, 1986b; Takehara, LeRoy & Grotendorst, 1987; Roberts *et al.*, 1988).

Inhibition of cytokine production is also an unlikely explanation for the action of antibodies to TLiSA1 in preventing the differentiation of CTL and LAK cells. It may be of importance therefore that TGFβ down-regulated TLiSA1 antigen expression and inhibited the IL-2- and TNF-α-mediated up-regulation of this antigen, even when added to pre-formed lymphoblasts. In this regard the effect of TGFβ upon TLiSA1 expression is the same as that reported for its effect on the Tac antigen (Kerhl *et al.*, 1986b), a finding confirmed during the present study; the expression of other, non-activation, antigens remains relatively unaltered by TGFβ.

In the present study it is shown that for maximum effectiveness, TGFβ must be present from the beginning of culture, and the same is true for anti-TLiSA1 antibodies (Burns *et al.*, 1985). Further, TGFβ has also been reported to significantly depress the function of NK cells (Rook *et al.*, 1987), and in a separate study we have found that rabbit antibodies to TLiSA1 inhibit the development of Tac-negative NK cells and also the increased function of NK leukaemia cells in response to IL-2 (which up-regulated TLiSA1 expression on these cells) (B. Jin, J. L. Scott and G. F. Burns, unpublished data). None of these findings provide unequivocal evidence for the nature of TLiSA1 but they suggest that the antigen is involved in differentiation events, perhaps involving receptor function. The facts that TGFβ profoundly modulates TLiSA1 expression and also inhibits the differentiation of cytotoxic effector cells in a similar way suggests that these events may be linked, and that the regulation of differentiation may be separated from the controls on proliferation.

The precursor of LAK cells is still a matter of some controversy, with evidence supporting a NK cell origin (Itch *et al.*, 1985; Phillips & Lanier, 1986; Ortaldo, Mason & Overton, 1986), and other data pointing to a T-cell origin (Burns *et al.*, 1984; Dambe, Doyle & Bradley, 1986; Kauffman *et al.*, 1987). Regardless of their origin it is likely that *in vivo* such cells provide a powerful non-specific amplification of the specific immune response, when they are generated in response to cytokines. A major role of TGFβ *in vivo* is now thought to be to promote wound healing and to suppress the local immune response at the sites of tissue repair. In establishing that TGFβ suppresses the development of LAK cells in culture, the present report complements reports of the immunosuppressive effects of TGFβ on NK cells, B cells and T cells in substantiating this theory. Further insights as to the mechanism of this inhibitory effect await the outcome of studies of the structure of TLiSA1 and its involvement in receptor function.

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