INHIBITION OF CYTOKINE PRODUCTION BY CYCLOSPORIN A AND TRANSFORMING GROWTH FACTOR β

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Cyclosporin A (CsA) has proven effective as an inhibitor of a variety of T cell responses in vitro, including antigen-specific proliferation, macrophage-mediated antigen presentation, and antigen- and lectin-induced cytokine production, including IL-1, IL-2, IL-3, and IFN-γ, but not IFN-α/β (1). CsA appears to function in the prevention of graft rejection by affecting the early activation of immunocompetent cells, possibly by blocking antigen-specific receptor binding, class I and II expression, inhibition of phospholipase A2 activity, and/or inhibition of synthesis of cytokine mRNA (1–4). The selective suppressive activity of CsA for cell-mediated immunity prompted its use in human organ transplantation (5).

Transforming growth factors (TGFs) are a family of peptides that, under certain conditions, can induce normal cells to express a transformed phenotype (6). At least two types of TGFs have been described: TGF-α, which is homologous to epidermal growth factor (EGF), and TGF-β. Mature TGF-β is a 25 kD homodimer held together by disulfide bonds that binds to a specific receptor(s) distinct from the TGF-α or EGF receptor (6). TGF-β inhibits IL-2-induced T cell proliferation, IL-2-R induction, IL-1-induced thymocyte proliferation, IFN-α but not IL-2-induced NK activity, B cell proliferation to growth factors and IFN-γ-induced class II antigen expression on Hs294T melanoma cells (7–11).

In this report, we describe the effects of CsA and TGF-β on the production of TNF-α (also referred to as cachectin), TNF-β (lymphotoxin), and IFN-γ by human PBMC and murine peritoneal macrophages. Our results demonstrate that CsA and TGF-β have suppressive effects on cytokine production that are dependent on their selective activity on specific cell types.

Materials and Methods

Cytokines. Recombinant human IFN-γ (rHuIFN-γ) and murine IFN-γ (rMuIFN-γ), cloned and expressed in *Escherichia coli* (12, 13), were purified to >99% purity (Rinderknecht, E., unpublished results). Specific activity of both IFNs was 1–2 × 10⁷ U/mg protein.

Isolation and Treatment of Mononuclear Cell Populations.   PBMC were isolated from buffy coats (Peninsula Blood Bank, Burlingame, CA) by Ficoll-Hypaque density sedimentation. The purified PBMC (2×10^6 cells/ml) were seeded into 24-well tissue culture plates (Costar, Cambridge, MA) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) (CRPMI). PBMC were pretreated with various doses of porcine platelet-derived TGF-β (>97% pure, R and D Systems Inc., Minneapolis, MN) for different time periods at 37°C, after which PHA-P (Difco Laboratories Inc., Detroit, MI) was added at a final concentration of 20 μg/ml. CsA (Sandoz, East Hanover, NJ) was added at various doses either simultaneously or 20 h before addition of 20 μg/ml PHA-P.

Adherent human monocytes were isolated by seeding PBMC into 24-well tissue culture plates (4×10^6 cells/well) and incubated for 90 min at 37°C. Nonadherent cells were removed and the adherent cells were washed three times in RPMI 1640 medium before addition of CRPMI. After 20 h of incubation with various concentrations of TGF-β, 1 μg LPS/ml (*E. coli* LPS 026:B6, Sigma Chemical Co., St. Louis, MO) and 1,000 U/ml rHuIFN-γ were added. Supernatants were removed for TNF-α ELISA assay 4 h later.

Murine peritoneal macrophages were obtained from 6–8-wk-old female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA). Peritoneal exudate cells (PEC) obtained from mice injected intraperitoneally 5–7 days previously with 0.5 ml thioglycolate (Difco Laboratories Inc.) were resuspended in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were incubated in plastic petri dishes for 1 h at 37°C. The adherent macrophages were scraped off gently and added to 12-well tissue culture plates (Costar) at 5×10^5/well with various concentrations of TGF-β. After 4 h of incubation with TGF-β, 5 μg/ml LPS and 100 U/ml rMuIFN-γ were added. Supernatants were removed after 72 h and were assayed for murine TNF-α activity in the L-M bioassay (14).

Cytokine Assays.   Human TNF-α and IFN-γ levels were determined by specific ELISA assays: The TNF-α ELISA has a detection limit of 40 pg/ml and the IFN-γ ELISA 0.4 ng/ml (15, 16). Human TNF-β and murine TNF-α were assayed on the L-M bioassay with a detection limit of 4 U/ml (14). To determine the level of TNF-β, all TNF-α activity was first neutralized by adding a threefold excess of a rabbit polyclonal anti-TNF-α antibody (17) for at least 30 min before assay. This antiserum has a neutralizing titer of 2.9×10^4 L-M U/μl. In the L-M bioassay, rHu TNF-β has a specific activity of 2×10^8 U/mg protein, and rHuTNF-α, 4×10^7 U/mg protein.

Results

Effects of CsA and TGF-β on Cytokine Production.   We examined the effects of CsA and TGF-β on TNF-α, TNF-β, and IFN-γ production by various PBMC populations. As shown in Fig. 1A, CsA, in a dose-related manner, inhibited the production of all three cytokines produced by PHA-P-stimulated unseparated PBMC cultures. CsA was more effective in inhibiting TNF-β and IFN-γ production compared with TNF-α (Fig. 1A), although a similar level of inhibition of TNF-α production could be obtained at a 10-fold higher CsA concentration (data not shown).

TGF-β also inhibited the production of IFN-γ in a dose-related manner, with maximal inhibition observed at 10 ng/ml (Fig. 1B). To obtain maximal inhibition of IFN-γ production, it was necessary to pretreat the PBMC for at least 4 h with TGF-β before the addition of PHA-P, although the addition of TGF-β at the initiation of culture resulted in significant inhibition (data not shown). In contrast to the inhibitory effects of CsA, TGF-β only minimally inhibited TNF-α and TNF-β production by PHA-P-stimulated unseparated PBMC (Fig. 1B). Trypan blue exclusion analyses of untreated and either CsA- or TGF-β-treated PBMC
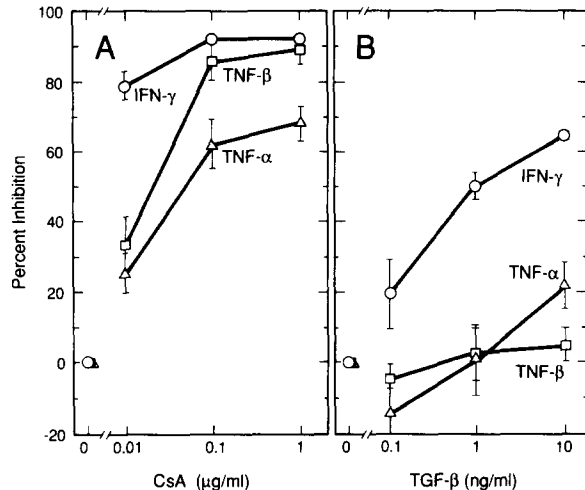
FIGURE 1. Effects of CsA (*A*) and TGF-*β* (*B*) on inhibition of cytokine production by unseparated PBMC. CsA was added together with 20 μg/ml PHA-P. TGF-*β* was added 4 h before PHA-P. TNF-*α* (△), TNF-*β* (□) and IFN-*γ* (○) levels were determined after 72 h of incubation. Results are presented as mean percent inhibition ± SEM (*n* = 6 for *A* and 5 for *B*). Mean cytokine production from untreated cultures was 1,431 pg/ml for TNF-*α*, 417 U/ml for TNF-*β*, and 21 ng/ml for IFN-*γ*.

cultures demonstrated similar cell viabilities (data not shown), suggesting that the inhibitory effects were not mediated by a reduction in cell viability but rather a suppression of functional abilities.

It was also demonstrated that anti-CD3 antibodies enhanced TNF-*α* and IFN-*γ* production by unseparated PBMC, and in support of the data with PHA-P stimulation, TGF-*β* significantly inhibited IFN-*γ* but not TNF-*α* production in the anti-CD3-stimulated cultures (data not shown). Since TNF-*α* is produced by various lymphoid populations (17, 18), we compared the ability of CsA and TGF-*β* to regulate TNF-*α* production by adherent, nonadherent, and unseparated PBMC cultures. TNF-*α* production by nonadherent PBMC was more susceptible to inhibition by CsA as compared with adherent PBMC and that the nonadherent cells (which contain both NK and T cells) produce TNF-*α* (Table I). In contrast, TGF-*β* inhibited TNF-*α* production by adherent PBMC over a range of concentrations tested (0.1–10 ng/ml), whereas TNF-*α* production by the unseparated PBMC was not significantly inhibited even at the highest concentration. In addition, TGF-*β* significantly enhanced TNF-*α* production at 1.0 and 0.1 ng/ml. A similar enhancement of TNF-*α* production by unseparated PBMC at subnanogram concentration of TGF-*β* was observed in 8 of 15 donors tested (data not shown).

The finding that TGF-*β* inhibited TNF-*α* production by human monocytes was further confirmed in murine macrophages (Table I), indicating that TNF-*α* production by monocytes/macrophages is particularly sensitive to the inhibitory effects of TGF-*β* and that the immunoregulatory activities of TGF-*β* are not species specific. Moreover, TGF-*β* significantly inhibited macrophage-mediated tumor cell cytostasis/cytotoxicity of NIH-3T3 target cells (data not shown).

Discussion

CsA, a widely used immunosuppressive agent, is a potent inhibitor of IL-1, IL-2, IFN-*γ*, and IL-3, as well as TNF-*α* and TNF-*β* production by human PBMC cultures (1–4). TNF-*α* has now been shown to exert a variety of immunoregulatory activities, such as stimulation of class I and class II antigen expression and

TABLE 1

Comparative Effects of TGF-β on TNF-α Production by Various Mononuclear Cell Populations

Exp.	Mononuclear cell populations*	Treatment	TNF-α ± SE	Change
			pg/ml	%
1	PBMC (nonadherent)	—	13,134 ± 1,501	—
		1 μg/ml CsA	1,480 ± 159	−88
	PBMC (adherent)	—	1,736 ± 144	—
		1 μg/ml CsA	1,458 ± 40	−14
		10 ng/ml TGF-β	748 ± 19	−57
		1 ng/ml TGF-β	1,283 ± 18	−26
		0.1 ng/ml TGF-β	1,380 ± 18	−21
	PBMC (unseparated)	—	4,550 ± 16	—
		10 ng/ml TGF-β	3,986 ± 192	−12
		1 ng/ml TGF-β	6,326 ± 66	+40
		0.1 ng/ml TGF-β	6,339 ± 19	+40
2	Murine peritoneal macrophages	—	253 ± 33[‡]	—
		10 ng/ml TGF-β	74 ± 5	−71
		1 ng/ml TGF-β	100 ± 20	−60
		0.1 ng/ml TGF-β	164 ± 21	−35

* Nonadherent, adherent, and unseparated PBMC were pretreated for 24 h with CsA or TGF-β before addition of stimulus. Nonadherent and unseparated PBMC were stimulated with 20 μg/ml PHA-P for 48 h and adherent PBMC were stimulated with 1 μg/ml LPS and 100 U/ml rHuIFN-γ for 4 h before supernatants were assayed for TNF-α levels. Murine peritoneal macrophages were pretreated for 4 h with TGF-β before addition of 5 μg/ml LPS and 100 U/ml rMuIFN-γ for 72 h.

‡ Results are presented as U/ml.

IL-1 production, as well as its involvement in cytotoxic T cell generation (19–20) (Ranges et al., unpublished observations). These results suggest that one important mode of action of CsA as an immunosuppressive agent may also be mediated through the inhibition of TNF-α production.

Recently, TGF-β has also been shown (7–11) to be a potent immunosuppressive agent in a variety of immunological systems. Our results demonstrate that TGF-β is a potent inhibitor of IFN-γ production by PBMC cultures. TGF-β inhibits T cell proliferation, probably by downregulating the expression of IL-2-R (7). The mechanism by which TGF-β inhibits NK-mediated killing is not known, but it has been suggested that TGF-β may inhibit the low levels of IFN produced in lymphocyte cultures that are important in the continuous recruitment and activation of pre-NK cells (9). Our observation that IFN-γ production by PBMC was inhibited by TGF-β suggests that TGF-β may suppress immune responses by inhibiting cytokine production.

It is unclear why TGF-β inhibits IFN-γ but not TNF-β production, both of which are products of lymphocytes. After mitogenic stimulation of PBMC, IFN-γ production precedes the production of TNF-β (18). In addition, recent studies have demonstrated that TGF-β can inhibit cytotoxic T lymphocyte as well as lymphokine activated killer cell development in vitro (Ranges et al.; Espevik, T., et al., unpublished observations). Presumably the failure of TGF-β to significantly inhibit TNF-β production, a relatively late-occurring event, indicates that TGF-β affects early stages of lymphoid cell maturation.

Although the immunosuppressive action of CsA seems to be most pronounced on T cells, previous studies (1) have shown that CsA can also affect macrophage functions. TNF-α production by adherent monocyte–enriched PBMC cultures

was less inhibited by CsA as compared with nonadherent lymphocyte–enriched PBMC cultures. In contrast, our results demonstrate that TNF-α production by adherent monocyte–enriched PBMC cultures was inhibited by TGF-β to a greater extent than in unseparated PBMC. These results suggest that TGF-β may primarily affect TNF-α production in monocytes, whereas CsA primarily inhibits TNF-α production in nonadherent lymphoid cells.

The significance of the finding that TGF-β suppresses cytokine production may be of importance in a homeostatic mechanism acting to limit cytokine-mediated inflammatory responses. This effect could occur through the release of TGF-β by activated platelets and T cells (7) at sites of inflammation and result in a reduction of immune cell cytokine production, class II antigen expression, and cytotoxic T cell development.

Summary

We investigated the ability of cyclosporin A (CsA) and transforming growth factor β (TGF-β) to modulate the production of TNF-α and TNF-β and IFN-γ by unseparated, nonadherent, and adherent PBMC. Treatment of unseparated PBMC with CsA resulted in a significant dose-dependent inhibition of all three cytokines ranging from >90% inhibition for IFN-γ and TNF-β, to ~70% for TNF-α. Pretreatment of unseparated or nonadherent PBMC with TGF-β inhibited the production of IFN-γ by 60–70%. However, the inhibition of TNF-α and TNF-β production by these cells was only minimally affected, and at 0.1–1 ng/ml TGF-β could enhance TNF-α production by unseparated PBMC. In contrast, pretreatment of adherent PBMC with TGF-β inhibited the production of TNF-α by ~60%. TGF-β also inhibited both TNF-α production and tumor cell cytotoxicity mediated by murine peritoneal-derived macrophages. These observations indicate that the biological effects of CsA and TGF-β on immune functions are of a wider range than previously reported.

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