Diltiazem Downregulates IL-12 Production by Human Dendritic Cells

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IT is well known that IL-12 plays a central role in the initiation and control of allogeneic immune response. It promotes the proliferation of lymphocytes and NK cells, cytotoxic activity of NK cells, and CTL. It was recently shown that IL-12 is involved in the regulation of T helper Th1-Th2 responses by exerting stimulatory effects on Th1 and inhibitory effects on Th2. This regulatory role is believed to result from the ability of IL-12 to induce IFN-γ production in activated T cells and NK cells.[1,2] Th1 cytokines (IL-2 and IFN-γ) promote both CTL and delayed-type hypersensitivity (DTH) responses, which are considered the principal effector mechanisms of allograft rejection.

Diltiazem, a calcium channel blocker used in organ transplantation, is often included in clinical protocols in association with cyclosporin A and corticosteroids.[3] It was used initially because of its antinephrotoxic and antihypertensive effects, so that the undesirable side effects induced by immunosuppressive therapy could be reduced.

We previously studied the effect of diltiazem on human mixed lymphocyte reactions (MLR) and on isolated human monocytes, showing the capacity of this drug to affect proinflammatory cytokine production.[4,5] Since dendritic cells (DCs) are the most effective antigen-presenting cells (APCs) to prime naive T cells, we were interested in determining the influence of diltiazem on human DCs. Human DCs generated from peripheral blood monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) have been characterised as immature DCs. To become fully potent APCs, DCs must undergo maturation induced either by a proinflammatory signal such as lipopolysaccharide (LPS) or by interaction with CD40L expressed on activated T lymphocytes.[6] The ability of mature DCs to act as potent APCs is due to their high expression of MHC and costimulatory molecules and also to their production of cytokines, especially IL-12. Therefore, we determined the effect of diltiazem on cytokine production by human DCs with a particular interest in IL-1β, IL-6, TNF-α, and IL-12 production.

MATERIALS AND METHODS
Chemical and Reagents

Diltiazem hydrochloride (Dilzene, Sigma-tau, Rome) was supplied as a powder, dissolved in culture medium at concentrations of 10^{-6} and 10^{-4} mol/L, and added on the third day of culture. For all concentrations used, a possible cytotoxic effect of the drugs was excluded by evaluation cell viability with trypan blue.

Human Peripheral Blood Mononuclear Cell Isolation

Human peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Hypaque method from buffy coats from healthy blood donors (courtesy of the National Transfusion Center of the Italian Red Cross, Rome).

Generation of DCs From Peripheral Blood of Healthy Donors

The method for in vitro culture of human DCs has been already described.[7] Briefly, PBMC were separated on multistep Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden), and the light-density fraction from the 42.5% to 50% interface was recovered. The recovered monocytes, which were >90% pure as shown by flow cytometry with an anti-CD14 antibody (clone 144, mouse IgG2a), were cultured at 5×10^5/mL in RPMI–10% FCS supplemented with 50 ng/ml GM-CSF (Novartis, Basel, Switzerland) and 1000 U/mL IL-4 (Basel Institute of Immunology) at 37°C at 5% CO_2 for 6 days before use. Diltiazem at 10^{-4} and 10^{-6} mol/L was added on the third day of culture. LPS from *Escherichia coli* (Sigma Chemicals, Bornem, Belgium), used at a concentration of 2 μg/mL and CD40L (J558 myeloma cells transfected with CD40 and provided by P. Lane, Oxford, UK) in a CD40L/DC ratio of 1:5 were added for the last 40 hours of culture.

Enumeration of IL-12-Producing Cells

The enzyme-linked immunospot method (ELISPOT), used to enumerate cytokine-secreting cells, was performed as previously described.[8] Briefly, plates were coated with 0.1 ml/well mouse anti-human IL-12 (Genzyme, Cambridge, Mass), in carbonate-bicarbonate buffer, pH 9.7, overnight at 4°C. After blocking, triplicates of the cultured cells were added to the wells and incubated overnight at 37°C in a 5% CO_2 incubator. After removal of the cells, plates were incubated with an optimal dilution of rabbit anti-human IL-12 (Genzyme) and then washed and incubated for 90 minutes at 37°C with alkaline-phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma Chemicals, St. Louis, Mo). The cytokines secreted by single cells were visualised by the addition of a solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) and counted under a stereo microscope.

Lymphokine Measurement by ELISA

Supernatants of DC cultures were harvested after 72 hours in the presence or absence of diltiazem. IL-1β, IL6, TNF-α, and IL-12 production was detected in the culture supernatants using sandwich ELISA (R&D Systems, Minneapolis, MN), and the results were expressed as picograms per milliliter. The optimal cytokine production in vitro was assessed in kinetic experiments.

RT-PCR and Densitometric Analysis

For cytokine mRNA analysis, cells were cultured at 1×10^6/mL in the presence or absence of diltiazem at concentrations of 10^{-4} and 10^{-6} mol/L. Forty hours after the addition of CD40L or LPS to the culture, total cellular RNA was extracted using the Rneasy Kit (Quiagen). RNA was reverse-transcribed into cDNA as previously described.[9] GAPDH and IL-12 sets of primers were synthesised by MWG Biotech; the following pairs of primers were used. GAPDH: sense, 5'-GTC TTC ACC ATG GAG AAG GTC-3'; antisense, 5'-CAT GCC AGT GAG CTT CCC GTT CA-3'; IL-12 p40 sense 5'-CCA AGA ACT TGC AGC TGA AG-3'; antisense 5' TGG GTC TAT TCC GTT GTG TC3'. Ten microliters of cDNA were amplified by PCR in a total volume of 20 μL. The PCR mixture contained a final concentration of $1 \times$ PCR buffer (10 mmol/L Tris.HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl$_2$), 50 μmol/L dNTP, 0.1 μmol/L of the 5' and 3' primers, and 1 unit of *Taq* DNA polymerase (Promega Biotec). The reaction products were amplified for 30 cycles in an automatic DNA thermal cycler (Perkin-Elmer, Cetus Corp.) Each cycle consisted of three steps: denaturation at 94°C for 45 seconds, annealing at 60°C for 2 minutes, and primer extension at 72°C for 3 minutes. After 30 cycles an additional extension step was performed at 72°C for 7 minutes. To avoid contamination all the experiments included control PCR without cDNA. For densitometric analysis, the reaction products were electrophoresed through a 1.8% agarose (Bio-Rad, Richmond, Calif) gel. Ethidium bromide-stained gel was analyzed on a UV transilluminator and photographed with positive/negative 665-type film (Polaroid Corp., Cambridge, Mass). The negative was scanned by densitometry (Bio-Rad Multi Analyst) as previously described.[9] The IL-12 mRNA levels were normalised to the GAPDH mRNA level.

RESULTS

DCs were generated from peripheral blood monocytes cultured in GM-CSF plus IL-4 in the absence or presence of diltiazem, added on the third day of culture. After 4 days of culture DCs were stimulated with LPS and CD40L-transfected J558 cells for 40 hours, and the expression of several molecules was assessed by FACS analysis. The analysis of surface markers showed that DCs in the presence of diltiazem, when compared to cells cultured alone, expressed the same level of MHC class I and II molecules, CD80, CD86, and CD83.[10,11] CD14 was either low or negative in the different preparations. In the presence of maturation stimuli (LPS and CD40L) MHC class I and II molecules were downregulated compared to DCs cultured without diltiazem at 10^{-4} mol/L, whereas CD86, CD83 and CD14 were not affected (data not shown).

Activated DCs produce a large variety of inflammatory and regulatory cytokines.[12] DCs from the GM-CSF + IL-4 cultures, stimulated by CD40L for 40 hours, secreted high

Table 1. Diltiazem Reduces IL-12 p70 Production in Supernatants of DC Cultures

	Cytokine Production (pg/mL)		
	Exp. 1	Exp. 2	Exp. 3
DC + LPS	78	76	6
DC + LPS + diltiazem at 10^{-4} mol/L	7	5	<1
DC + LPS + diltiazem at 10^{-6} mol/L	51	32	<1
DC + CD40L	5010	10,130	3275
DC + CD40L + diltiazem at 10^{-4} mol/L	1940	4072	1450
DC + CD40L + diltiazem at 10^{-6} mol/L	5906	10,824	3520

levels of IL-12.[6] Interestingly, we demonstrated that diltiazem exerts a clear inhibitory effect on IL-12 production. Table 1 shows the downmodulation of diltiazem on the production of IL-12 as detected by ELISA. The range of inhibition was between 60% and 76% when diltiazem at 10^{-4} mol/L was used. On the contrary, IL-1β, IL-6, and TNF-α production was unaffected (Table 2).

Enumeration of cytokine-producing cells was detected by the enzyme-linked immunospot (ELISPOT) method. The results of the effect of diltiazem on DCs at the highest dose show a reduction in the number of IL-12-producing cells (Table 3).

To verify whether the inhibitory effect of diltiazem on IL-12 production by human DCs corresponded with a comparable effect on mRNA transcription, a semiquantitative reverse transcription polymerase chain reaction analysis was performed. Diltiazem, at 10^{-4} mol/L, clearly reduced the IL-12 p40 mRNA level both in the presence of LPS and of CD40L (Fig. 1).

These results indicate the capability of diltiazem to

Table 2. Diltiazem Has No Effect on IL-1β, IL-6, and TNF-α Production by Human Dendritic Cells

	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
DC	20	20	85
DC + diltiazem at 10^{-4} mol/L	18	14	79
DC + diltiazem at 10^{-6} mol/L	21	14	83
DC + LPS	43	1023	952
DC + LPS + diltiazem at 10^{-4} mol/L	31	1278	1115
DC + LPS + diltiazem at 10^{-6} mol/L	37	1120	1036
DC + CD40L	51	3950	3870
DC + CD40L + diltiazem at 10^{-4} mol/L	46	4030	3925
DC + CD40L + diltiazem at 10^{-6} mol/L	42	4032	3889

One representative experiment out of three is reported.

**Table 3. Diltiazem Reduces the Number of
IL-12-Producing Cells**

	Spot-forming cells		
	Exp. 1	Exp. 2	Exp. 3
DC + LPS	121	7	47
DC + LPS + diltiazem at 10^{-4} mol/L	17	3	33
DC + LPS + diltiazem at 10^{-6} mol/L	78	5	47
DC + CD40L	421	225	183
DC + CD40L + diltiazem at 10^{-4} mol/L	100	62	81
DC + CD40L + diltiazem at 10^{-6} mol/L	321	70	97

inhibit IL-12 production by DCs that have been induced to maturation by different stimulation.

DISCUSSION

Diltiazem is a calcium antagonist drug used in kidney transplantation in association with other conventional immunosuppressive drugs during the first week after transplantation to prevent acute rejection. Its effect on T and B lymphocytes, NK cells, and MLR cultures has been well established;[13] diltiazem, in fact, shows a clear immunosuppressive function when used alone, and an immunomodulatory capability when used in association with cyclosporin A and corticosteroids.[3] We recently showed that diltiazem

Fig 1. Effect of 10^{-4} mol/L diltiazem on IL-12 p40 mRNA levels in human dendritic cells. Total cellular RNA was extracted and amplified by RT-PCR. Lane 1, untreated DC; lane 2, DC plus diltiazem; lane 3, DC plus LPS; lane 4, DC plus LPS treated with diltiazem; lane 5, DC stimulated with CD40L-transfected cells; lane 6, DC stimulated with CD40L-transfected cells and treated with diltiazem. Results are also expressed as arbitrary units calculated with the IL-12 p40 mRNA and GAPDH mRNA ratio. One representative experiment out of three is shown.

acts on isolated human monocytes as well, affecting monokine production[5] and nitric oxide (NO) levels.[14]

In this study we investigated the effect that diltiazem might exert on human dendritic cells, focusing on cytokine production. IL-1β, IL-6, TNF-α, and IL-12 levels were assessed since we had previously demonstrated an increase in IL-1β production by monocyte cultures in the presence of the drug. An interesting effect of diltiazem on DCs has been herein demonstrated using different approaches, which clearly show that diltiazem reduces IL-12 production by reducing IL-12 transcription.

The inhibition of IL-12 synthesis could favour Th2 polarisation of CD4$^+$ T-cell responses, and we could speculate that a preferential induction of Th2 would promote graft acceptance.

The role of IL-12 in transplant rejection remains unclear. A study shows that reduction of IL-12 by neutralisation with polyclonal anti-IL-12 antibodies results in the amelioration of acute graft-versus-host disease (GVHD) and, conversely, that treatment with exogenous IL-12 converts chronic GVHD into exacerbated acute GVHD.[15] On the other hand, a recent evidence questions the validity of the Th1/Th2 paradigm since IL-12 antagonism exacerbates cardiac allograft rejection yet promoting intragraft Th2 cytokines gene expression. In vivo Th1 priming is not inhibited by IL-12 neutralisation, indicating that Th1 development may occur independently of IL-12.[16]

Downregulation of IL-12 seems to be an important step in the action of immunosuppressive drugs since some reports showed that cyclosporin A and glucocorticoids reduce IL-12 production by DC.[17,18] The decrease in IL-12 production caused by diltiazem, cyclosporine, and corticosteroids might explain the primary inhibition of Th1-type alloresponses by these drugs.

These findings confirm that antigen-presenting cells are among the targets of calcium channel blockers and extend our knowledge of the mechanism of action of these drugs. The effect of diltiazem on IL-12 production, added to its antiproliferative capability and antihypertension activity, may encourage more frequent clinical use in transplantation in association with other immunosuppressive drugs.

Studies on further possible effects on other DC functions are under investigation and might provide additional information on the effect of calcium antagonist drugs on the immune system.

REFERENCES

1. Gately MK, Renzetti LM, Magram J, et al: Annu Rev Immunol 16:495, 1998

2. Trinchieri G: Annu Rev Immunol 13:251, 1995

3. D'Ambrosio A, Segoloni G, Quintieri F: Transplant Int 10:426, 1997

4. D'Ambrosio A, Giacomini E, Camponeschi B, et al: Transplantation 65:1411, 1998

5. D'Ambrosio A, Giacomini E, Quaranta MG, et al: Transplant Proc 30:4188, 1998

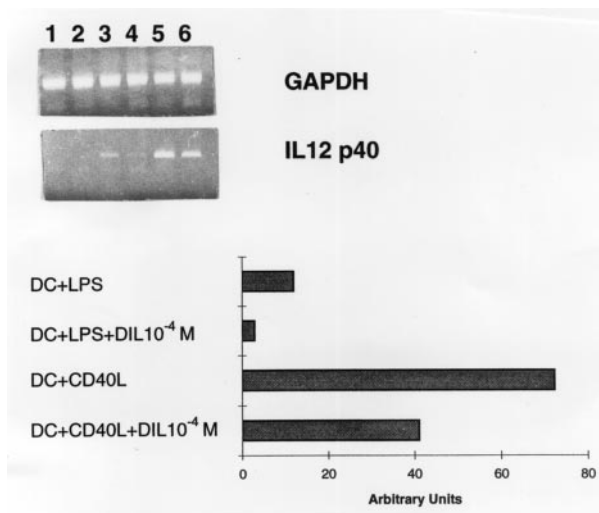6. Cella M, Scheidegger D, Palmer-Lehmann K, et al: J Exp Med 184:741, 1996

7. Sallusto F, Lanzavecchia A: J Exp Med 179:1109, 1994

8. Luzzati AL, Boirivant M, Giacomini E, et al: Clin Exp Immunol 105:403, 1996

9. Viora M, Camponeschi B: Cell Immunol 163:289, 1995

10. Banchereau J, Steinman RM: Nature 392:245, 1998

11. Cella M, Sallusto F, Lanzavecchia A: Curr Opin Immunol 9:10, 1997

12. de Saint-Vis B, Fugier-Vivier I, Massacrier C, et al: J Immunol 160:1666, 1998

13. Marx M, Weber M, Merkel F, et al: Nephrol Dial Transplant 5:1038, 1990

14. D'Ambrosio A, Giacomini E, Bachetoni A, et al: Transplant Proc, in press, 2001

15. Williamson E, Garside P, Bradley JE, et al: J Immunol 157:689, 1996

16. Piccotti JR, Chan SY, Goodman RE, et al: J Immunol 157:1951, 1996

17. Bonnotte B, Pardoux C, Bourhis JH, et al: Tissue Antigen 48:265, 1996

18. Vanderheyde N, Verhasselt V, Goldman M, et al: Transplantation 67:1342, 1999