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ORIGINAL ARTICLE

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The modulatory effect of diltiazem on human in vitro alloreactivity when used alone or in combination with cyclosporin A and/or methylprednisolone

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Abstract The calcium channel blocker diltiazem is often included in post-transplant regimens in combination with other immunosuppressive drugs such as cyclosporin A (CyA). It is primarily used because of its antinephrotoxic and antihypertensive effects, so that undesirable side effects induced by the immunosuppressive therapy can be reduced. Its alleged ability to induce direct immunosuppression may explain the encouraging results from its clinical use and would appear to encourage a much wider use of this drug. The present study shows the effect of diltiazem on the human in vitro alloresponse when used alone or in combination with cyclosporin A (CyA) and methylprednisolone (MP). The results show that, when administered alone, diltiazem exerts a suppressive effect, but only at high, non-therapeutic doses. Interestingly, in combination with CyA or MP, diltiazem enhances the suppressive effect of these two drugs on in vitro alloresponses at lower doses. This additional effect of diltiazem may contribute to better graft survival in clinical transplantation.

Keywords Diltiazem, alloreactivity · Immunosuppression, diltiazem · MLR, diltiazem

Introduction

Cyclosporin A (CyA) is universally considered an essential drug after organ transplantation. It is currently administered in combination with corticosteroids in the postoperative treatment of transplant recipients. Nevertheless, its immunosuppressive efficacy is limited by several side effects, i.e. its chronic or acute nephrotoxicity and its hypertensive effects, which only add to the well-known side effects of the steroids. Many attempts have been made to diminish the nephrotoxic effect by combining CyA with new classes of drugs [1, 10]. Calcium channel blockers have been widely used in clinical regimens together with CyA [9, 18, 22]. Diltiazem is one of the most promising calcium antagonist drugs, not only because of its antinephrotoxic and antihypertensive effects, but also because of its alleged ability to provide immunosuppression, as evidenced by a lower rate of organ rejection in patients treated with this drug [6, 7, 12, 19]. In particular, diltiazem can induce higher blood levels of CyA since it decreases the clearance of intravenously administered CyA and exerts an immunosuppressive effect through its metabolites [14] and/or a likely direct effect [16]. In contrast, the effect of diltiazem when combined with corticosteroids has not yet been established.

The aims of the present study were to demonstrate the direct immunosuppressive effect of diltiazem on the human in vitro alloresponse and, most importantly, to study its effect when administered together with CyA and/or methylprednisolone (MP). Since clinical regimens for the prevention of organ rejection routinely include CyA and/or corticosteroids [11], we attempted to find low-dose drug combinations that were capable of producing a high immunosuppressive effect while also reducing cytotoxicity and improving cost benefits.

Materials and methods

Chemicals and reagents

Cyclosporin A (CyA; Sandimmun, Sandoz Wander Pharma, Bern, Switzerland) was supplied as a sterile, purified, aqueous solution at a concentration of 50 mg/ml and used at 0.01, 0.1, and 1 μg/ml. Diltiazem hydrochloride (Angizem, Inverni della Beffa, Milan, Italy) was supplied as a powder, dissolved in culture medium, and used at 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M. Finally, methylprednisolone sodium succinate (MP; Urbason, Hoechst, Milan, Italy) powder was dissolved in water for injection at a concentration of 20 mg/ml and used at 0.01, 0.1, and 1 μg/ml. All stock solutions were prepared on the day of the experiment and the serial dilutions were made with the culture medium. In all of the experiments, the drugs were added at the beginning of cultures.

Human lymphocyte isolation

Human peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Nycomed, Oslo, Norway) gradient separation of buffy coats from healthy blood donors, courtesy of the National Transfusion Center of the Italian Red Cross (Rome). All human studies were approved by the appropriate ethics committee and were performed in accordance with the ethical standards set down in the 1964 Declaration of Helsinki. All participants gave their informed consent prior to their inclusion in the study.

Alloreactive cell line preparation

PBMC were stimulated with irradiated (3000 rads) allogeneic PBMC at a ratio of 1:2 in culture medium RPMI 1640, supplemented with 10% human serum (PAA Laboratory, Linz, Austria), L-glutamine, and penicillin/streptomicin, in 75 cm^2 flasks (Falcon 3024). After a week of incubation at 37 °C in a humidified atmosphere at 5% CO_2, blasts were collected and used for the assay.

Proliferative assay

Blasts (1×10^5) were stimulated with an equal number of irradiated allogeneic PBMC in flat-bottomed, 96-well microtiter plates in the presence of different doses of the drugs alone or in combination. At day 4, after the addition of 0.5 μCi of ^{3}H-thymidine for the last 20 h, cultures were harvested and [^{3}H] determinations were done using an LKB beta-plate spectrometer (Pharmacia LKB Biotechnology, Turku, Finland). Results were expressed as counts per minute (cpm) + standard deviation (SD). The percentage inhibition of lymphocyte proliferation at each dose of drugs alone or in combination was calculated using the formula:

$$\%\,\text{Inhibition} = 1 - \frac{\text{cpm in drug presence}}{\text{cpm in drug absence}} \times 100$$

Statistical analysis

Results were expressed as mean ± SEM and compared using Student's *t*-test for paired data. *P* values below 0.05 were considered significant.

Results

Previous experiments have indicated that 0.01 μg/ml of both CyA and MP is the dose that best fits our in vitro system (data not shown). For this reason, this dose was used in the starting experiments.

Absolute values of cell proliferation inhibition are different because of individual variability [21]; no clear influence of HLA phenotype has been found by Zlabinger et al. [26].

The effect of diltiazem 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M on the alloreactive response of normal human PBMC, after a second stimulation with allogeneic cells, is shown in Fig. 1. Concentrations higher than 10^{-4} M were not used since Birx et al. [2] demonstrated that, at concentrations greater than 5×10^{-4} M, the drug was found to be consistently cytotoxic. For all of the concentrations used, a possible cytotoxic effect of the drugs was excluded using evaluation of cell viability with trypan blue. The inhibitory effect of diltiazem is dose-dependent, reaching the highest values at 10^{-4} M. Concentrations between 10^{-7} and 10^{-6} M have been shown to correspond to therapeutic dosages [5, 13].

The combination of different concentrations of diltiazem with CyA 0.01 μg/ml is shown in Fig. 2. The dose of CyA is below the range of plasma and blood concen-

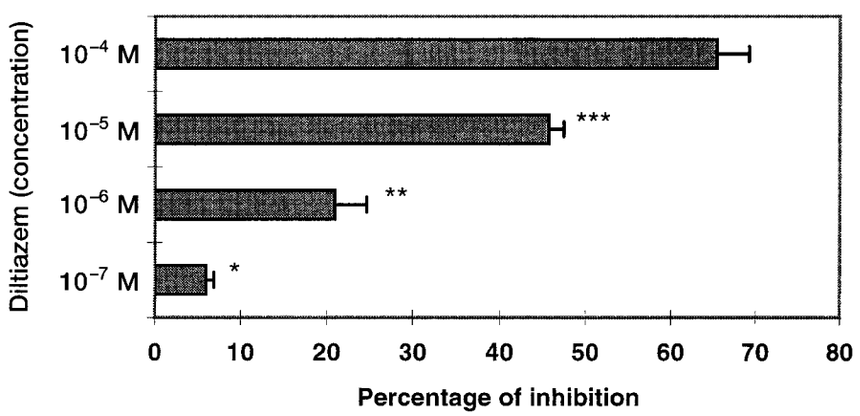

Fig. 1 Immunosuppressive effect of diltiazem at different concentrations in MLR. Results are expressed as mean ± SEM of four experiments. *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$ versus diltiazem 10^{-4} M (Student's *t*-test for paired data)

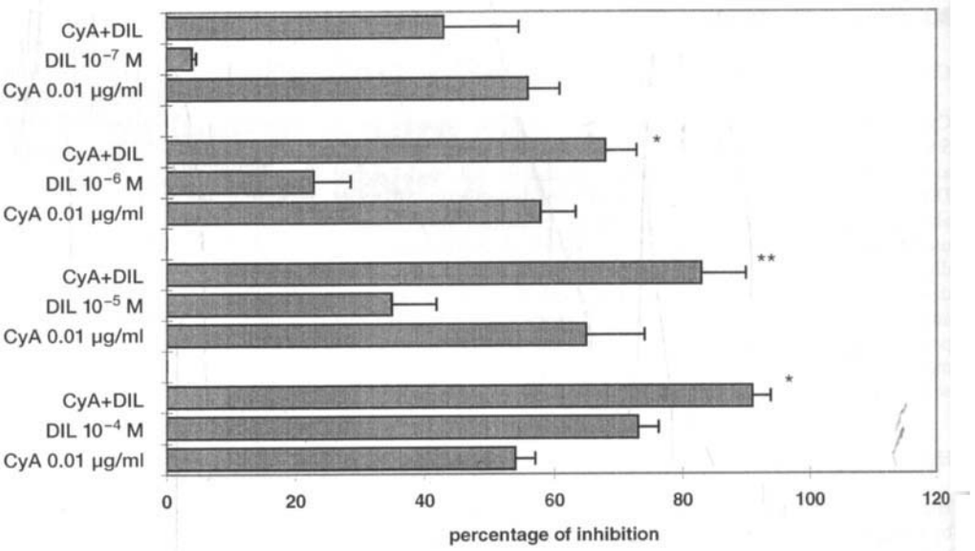

Fig. 2 Diltiazem (DIL) modulates the immunosuppressive effect of cyclosporin (CyA) 0.01 µg/ml in MLR at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M. Results are expressed as mean ± SEM of four experiments. $*P < 0.005$; $**P < 0.05$ versus CyA 0.01 µg/ml (Student's *t*-test for paired data)

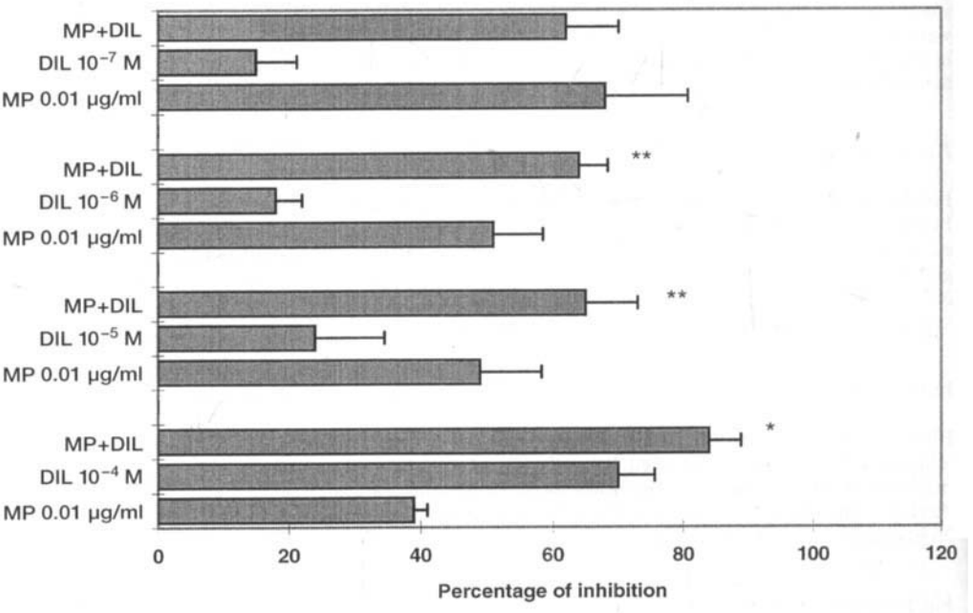

Fig. 3 Diltiazem (DIL) modulates the immunosuppressive effect of methylprednisolone (MP) 0.01 µg/ml in MLR at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M. Results are expressed as mean ± SEM of three experiments. $*P < 0.005$; $**P < 0.05$ versus MP 0.01 µg/ml (Student's *t*-test for paired data)

trations in patients; this dose was chosen because in our system it shows an immunosuppressive activity of around 50 %. In all combinations except that with the lowest concentration of diltiazem (10^{-7} M), the two drugs show an increase in inhibition of the proliferative response in comparison to the effects of the two drugs alone. In particular, diltiazem 10^{-6} and 10^{-5} M show a better additional suppressive effect in combination with CyA than diltiazem 10^{-4} M, which exerts a more suppressive effect when used alone.

The combination of the same concentrations of diltiazem with 0.01 µg/ml MP is shown in Fig. 3. MP is used at a therapeutic concentration that lies within the range of maintenance steroid therapy and results in about 50 % inhibition of proliferative alloresponse in vitro [3]. Once again, diltiazem 10^{-6} and 10^{-5} M exert the best additive effect; these data are very interesting given the fact that doses between 0.1 and 0.7×10^{-6} M correspond to in vivo therapeutic concentrations of diltiazem [5].

Since a combination of three immunosuppressive drugs is widely used in clinical regimens for preventing organ rejection in transplantation, a combination of different doses of diltiazem with CyA and MP, both at the

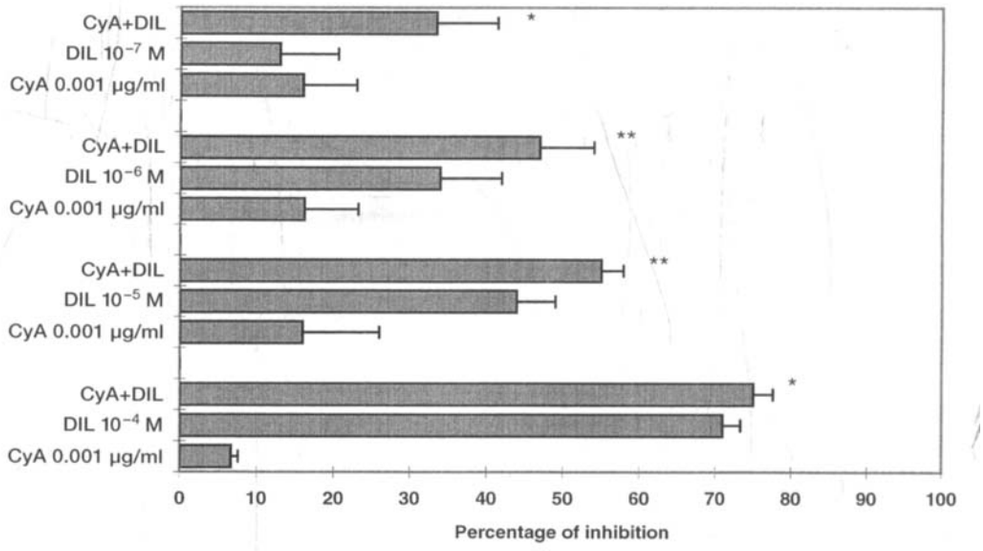

Fig. 4 Low concentrations of diltiazem modulate better when added to a subtherapeutic dose of CyA (0.001 μg/ml). Results are expressed as mean ± SEM of four experiments. $*P < 0.005$; $**P < 0.05$ versus CyA 0.001 μg/ml (Student's *t*-test for paired data)

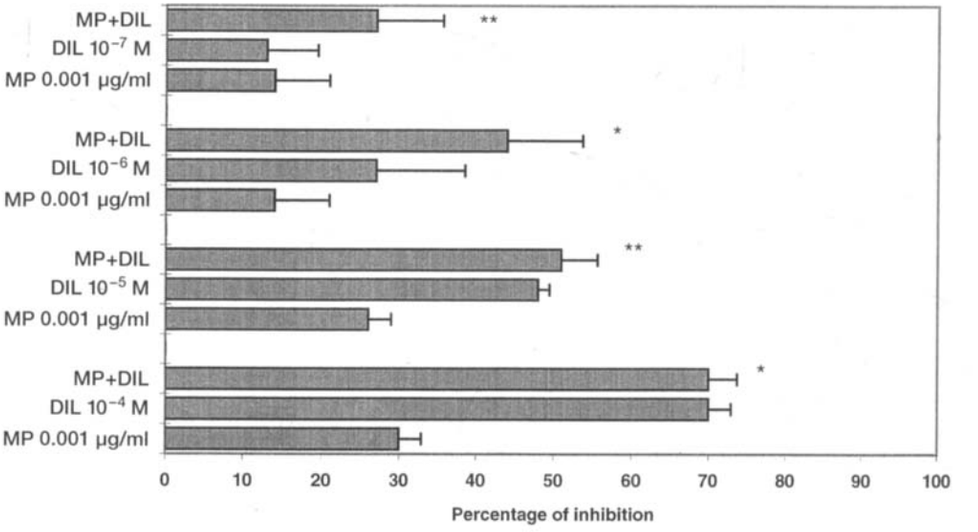

Fig. 5 Low concentrations of diltiazem modulate better when combined with a subtherapeutic dose of MP (0.001 μg/ml). Results are expressed as mean ± SEM of four experiments. $*P < 0.005$; $**P < 0.05$ versus MP 0.001 μg/ml (Student's *t*-test for paired data)

concentration of 0.01 μg/ml, were also tested. The inhibitory effect of CyA and MP together on the alloreactive response is close to 100 %, so the addition of diltiazem at any dose is virtually unnecessary (data not shown). For this reason, lower concentrations of CyA and MP were tested in combination with all concentrations of diltiazem to detect any possible additional effect that could have been masked by the concentrations previously used. The suppressive effect of diltiazem with CyA or MP 0.001 μg/ml is given in Figs. 4 and 5. Interestingly, the addition of diltiazem 10^{-7} and 10^{-6} M induces a stronger inhibition than that with CyA or MP 0.01 μg/ml. Therefore, in our system, the combination of very low doses of the drugs seems to be more effective than all of the other combinations tested. Here, again, combining the three drugs yielded no clear results (data not shown).

Discussion

The present study shows the effect of the calcium antagonist diltiazem on the human in vitro alloresponse. Both the proliferation and the immunosuppression that we observed in our system showed an individual variability that seems not to depend on HLA phenotype, as previously described [21, 26].

First, we demonstrated a direct dose-dependent immunosuppressive effect of diltiazem in MLR. Since the most effective concentration of diltiazem exceeds the

range of therapeutic doses, we focused our study on the combination of lower doses of diltiazem with two other immunosuppressive drugs routinely used to prevent rejection after organ transplantation. In particular, the combination with CyA provided very interesting results because of diltiazem's ability to limit some of the side effects of CyA and to increase its clearance [23]. Our results using diltiazem alone and at low concentrations together with CyA confirm findings that clearly show a direct dose-response immunosuppressive effect and an additive inhibitory effect, respectively, in MLR [15]. Comparable results have been reported in similar studies on other calcium antagonists (nicardipine and verapamil) on murine spleen cells [17] and on human mononuclear blood cells [8, 24]. However, in vitro results using diltiazem and MP have not yet been reported, though glucocorticoids are widely included in immunosuppressive clinical regimens. Herein we show results using the combination of MP with diltiazem and/or CyA in our in vitro system.

We tested different concentrations of diltiazem (from 10^{-7} to 10^{-4} M) with subtherapeutic concentrations of CyA and MP in order to find the best combination capable of exerting a good immunosuppressive effect and, at the same time, of limiting the undesirable side effects. Combinations of diltiazem with CyA or MP 0.01 μg/ml showed an increased rate of immunosuppression with all of the molarities used, 10^{-7} M excepted. Interestingly, with still lower doses of CyA and MP (0.001 μg/ml), a positive immunomodulatory effect was achieved, even with diltiazem 10^{-7} M.

Many transplantation centers use triple therapy with CyA, azathioprine, and prednisolone, since the ideal immunosuppressive regimen should be a combination of drugs rather than a single agent. For this reason, we combined different doses of diltiazem with both CyA and MP, at low concentrations. In our system, the high inhibitory effect exerted by CyA and MP together masks any other possible immunosuppressive effect of diltiazem, suggesting the preferential use of the double, rather than triple, combination.

These data provide interesting information about doses and combinations of the three drugs and their potential use in therapy. They also definitely support the use of diltiazem in clinical regimens in combination with CyA or MP, even though the in vivo inhibitory effect of diltiazem and MP on CyA hepatic metabolism [4, 20] and its effect on patient outcome need to be taken into consideration.

Further experiments are needed to analyze the mechanism of action that allows diltiazem to exert its immunosuppressive effect. Of interest would be a study of the effect of diltiazem on the production and release of interleukins in MLR at different levels. Zanker et al. [25] demonstrated that the calcium antagonist verapamil suppresses the release of IL-2 and the proliferation and generation of cytotoxic T-cell activity in mitogen and alloantigen-stimulated human T lymphocytes through a modulatory effect at the transcriptional level.

In conclusion, this study shows that, when administered alone, the calcium antagonist diltiazem exerts a direct immunosuppressive effect only at high nontherapeutic doses; however, in combination with CyA or MP, diltiazem enhances the immunosuppressive effect of these two drugs on in vitro alloresponses at very low doses. These results suggest that satisfactory graft survival and a concomitant reduction in detrimental side effects may be achieved in clinical transplantation, even when drug concentrations are reduced.

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