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Gangliosides potentiate the immunosuppressive effects of cyclosporin A in rat skin allografts

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Abstract. In vitro gangliosides exert inhibitory effects on cellular immune responses, largely relying on an impairment of the IL-2/IL-2 receptor interaction. In a previous study we have demonstrated synergistic effects of gangliosides and cyclosporin A (CyA) in the inhibition of the generation of in vitro allospecific immune responses in humans. To evaluate the possibility of using these drugs in immunosuppressive therapy in organ transplantation, we investigated the effects of the combination of a gangliosides mixture (GAMIX) and suboptimal doses of CyA on rat skin allografts in vivo. Sprague-Dawley rats were implanted with skin grafts from Lewis rats and treated for 21 days by intraperitoneal administration of either GAMIX or CyA or a combination of the two drugs. Untreated, GAMIX-treated or CyA-treated rats rejected skin allografts. In contrast, when a combined GAMIX CyA treatment was administered, successful grafting could be obtained in 8 rats out of 10 tested. Cells derived from spleens on day 21 post graft were stimulated in vitro with PWM mitogen. We found that cells from transplanted rats, untreated or treated with low-dose CyA or GAMIX alone, showed comparable responses to PWM. Cells from rats treated with the combination of the two drugs were found to be virtually unresponsive to stimulation by PWM mitogen. Taken together, our results indicate that GAMIX potentiate in vivo and ex vivo immunosuppressive effects of low-dose CyA.

Key words: Cyclosporin A – Gangliosides – Immunosuppression – Skin allografts

The discovery and characterization of the pharmacological effects of cyclosporin A (CyA) has represented a major advance in the immunosuppressive treatment in clinical organ transplantation. CyA therapy reduced both number and severity of acute rejection episodes, thus markedly increasing the success rates in bone marrow, heart, pancreas, liver, and kidney transplants [2, 6, 9]. A major limitation in the clinical use of CyA, however, is represented by its toxicity [5, 6]. It is thus of interest to evaluate the possibility of potentiating the immunosuppressive effects of CyA by taking advantage of less toxic compounds.

Gangliosides are complex glycosphingolipids that have been reported to play a role in the control of cell proliferation and differentiation [1, 3] possibly related to an inhibition of protein kinase C activation by endogenous diacylglycerols [7], or mediated through the action of discrete ganglioside metabolites [4]. Furthermore, gangliosides have been shown by several authors, including ourselves, to exert immunosuppressive effects in vitro [8, 10, 12], largely due to an alteration in the binding of IL-2 to its receptors [10].

Considering the emerging clinical need for low-dose CyA immunosuppressive regimens, we previously investigated the synergic effects of these two drugs in in vitro inhibition of allostimulated cellular immune responses. We found that GAMIX, combined with low doses of CyA, can significantly potentiate the inhibitory effects of CyA on MLC and CTL generation. Furthermore, GAMIX and CyA efficiently synergize in inhibiting the production of IFN gamma induced in MLC [13].

In this study we sought to explore the possibility of combine gangliosides and suboptimal doses of CyA to obtain synergistic immunosuppression in rat skin allografts in vivo. We found that gangliosides potentiate the immunosuppression exerted by low doses of CyA both on skin rejections and on proliferative responses induced by mitogens in spleen lymphocytes from rats treated with the combined immunosuppressive therapy.

Materials and methods

Animals

Fortyh-three inbred strain Sprague-Dawley rats (Charles River, Italy) were used as recipients while inbred strain Lewis rats were used as skin graft donors. All animals

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Table 1. Effects of CyA and GAMIX immunosuppressive therapy on skin allograft rejection*

Groups	Treatment	Skin rejections/ no. rats
1	Skin autograft	0/5
2	Skin allograft	15/15
3	Skin allograft + CyA 2 mg/kg/day	0/5
4	Skin allograft + CyA 1 mg/kg/day	6/9
5	Skin allograft + CyA 0.5 mg/kg/day	13/14
6	Skin allograft + GAMIX 30 mg/kg/day	9/9
7	Skin allograft + GAMIX 30 mg/kg + CyA 1 mg/kg/day	1/5
8	Skin allograft + GAMIX 30 mg/kg + CyA 0.5 mg/kg/day	2/10

* $P < 0.001$, group 2 vs group 7; $P < 0.0001$, group 2 vs group 3 and group 8

were male with a mean age of 3–4 months and a mean weight of 300 g. The animals were maintained under standard laboratory conditions.

Surgical procedures

The rats were anesthetized intraperitoneally with ketamine, 30 mg/kg. The cervicodorsal area was prepared to obtain the graft or to implant it. A 2 cm^2 skin patch was removed by a full-thickness incision that included dermis and epidermis. The graft was then implanted and sutured in the cervicodorsal area of the recipient. Post-operatively the animals were kept in separate cages to avoid any possible damage to the graft.

Immunosuppressive treatment

Drugs. CyA (Sandoz, Basel, Switzerland) was dissolved in ethanol and further diluted in saline. CyA was injected intraperitoneally at doses ranging between 0.5 and 2 mg/kg per day. Bovine brain ganglioside mixture, here after referred to as GAMIX (Cronassial, FIDIA, Abano Terme, Italy), was composed as follows: GM1 21%, GD1a 40%, GD1b 16% and GT1b 19% weight/volume. GAMIX preparations were dissolved in saline and injected intraperitoneally at 30 mg/kg per day. Table 1 reports the experimental groups under study. The drugs were administered 5 days/week for a period of 3 weeks.

Proliferation studies. All rats were killed 21 days after the beginning of treatment and splenectomized. Spleen cells were resuspended at 1×10^6 in RPMI medium supplemented with glutamine (2 mM), 10% FCS and antibiotics (complete medium) and cultured in 96 flat-bottom microwell trays (Nunc, Denmark) in triplicate samples in the presence or absence of PWM (Sigma) for 3 days in humidified 5% CO_2 atmosphere. Proliferation was assessed by ^{3}H-thymidine incorporation following a 18-h pulse.

Statistical analysis. The significance of the differences of the means was analyzed by the one-tailed Student's *t*-test. The significance of the differences in skin rejections was analyzed by Fisher's exact test.

Results

Effects of CyA and GAMIX therapy on skin allograft rejection

Table 1 reports the data concerning the different groups of animals under study, the immunosuppressive treatments, the number of animals for each group, and the number of skin rejections recorded. In group 2 with no immunosuppressive therapy, group 5, treated with suboptimal doses of CyA, and group 6, treated with GAMIX alone, graft rejection was observed in all animals. The number of rejections was significantly ($P < 0.001$) lower in rats treated with CyA 2 mg/kg (group 3) or, more interestingly, in rats treated with GAMIX 30 mg/kg plus CyA 1 mg/kg (group 7) or GAMIX 30 mg/kg plus CyA 0.5 mg/kg (group 8).

Effects of CyA and GAMIX immunosuppressive therapy on mitogen induced spleen-cell proliferation

The effects of the combination of CyA and GAMIX were then studied on lymphocyte proliferation induced by PWM. Spleen cells from the different groups of animals under treatment were stimulated in vitro with an optimal dose of PWM (5 μg/ml). Table 2 shows the proliferation of spleen cells from group 2 – skin-recipient rats, group 5 – skin-recipient rats treated with CyA, group 6 – skin-recipient rats treated with GAMIX, or group 8 – skin-recipient rats treated with a combination of the two drugs. The data are reported as the mean of cpm ± SD values of spleen cell cultures from each rat (five animals in each group). Spleen cells from untreated or skin allografted animals showed an optimal proliferative response to PWM stimulation. Similarly, spleen cells from rats treated with CyA or GAMIX alone optimally proliferate following mitogen stimulation. In contrast, the proliferation of spleen cells from animals treated with a combination of CyA and GAMIX was significantly reduced compared to that of spleen cells from group 2 animals ($P < 0.03$). The proliferation induced by PWM was also significantly reduced ($P < 0.0001$) in spleen cells from rats treated simultaneously with CyA and GAMIX in comparison with spleen cells from animals treated with CyA alone.

Discussion

The data reported in this investigation indicate that gangliosides can augment in vivo the immunosuppression induced by low doses of CyA in skin-grafted rats. Indeed, in

Table 2. Effects of CyA and GAMIX immunosuppressive therapy on PWM-induced spleen-cell proliferation*

Groups	Treatment	^{3}H-Tdr incorporation (cpm × 10^{-3} ± SD)
	Untreated rats	40.4 ± 6.4
2	Skin allograft	42.2 ± 31.4
5	Skin allograft + CyA 0.5	27.3 ± 9.4
6	Skin allograft + GAMIX 30	35.5 ± 30.7
8	Skin allograft + GAMIX 30 + CyA 0.5	5.1 ± 6.8

* $P < 0.03$, group 2 vs group 8; $P < 0.001$, group 5 vs group 8

the group of rats treated with a combination of the two drugs, at the lowest dose of CyA used, a significant reduction in skin rejections could be observed. Moreover, spleen cells from animals treated with both drugs showed significantly decreased proliferative activity upon stimulation by PWM mitogen in vitro. These results are in accordance with our previous findings on the potentiation of immunosuppressive effects between CyA and GAMIX, in vitro, in the generation of allospecific immune response in man [13].

Still unclear is the nature of the mechanisms underlying this potentiation. As already reported [10, 12], gangliosides do not affect IL-2 production in vitro, in contrast to CyA, which exerts typical, dose-dependent, inhibitory effects. However, GAMIX can down-regulate IL-2 production in MLC performed in the presence of low doses of CyA. GAMIX does not influence Tac expression in MLC blasts, while again CyA shows dose-dependent inhibitory effects [13]. Considering the autocrine nature of IL-2 production [11], one suggestive hypothesis to explain the potentiation exerted by GAMIX on CyA-induced immunosuppression is that GAMIX might exert the immunosuppressive effects by inhibiting the interaction of IL-2 with its receptor [10] and thus prevent further IL-2 production. This mechanism might be of relevance in the presence of low-dose CyA, unable per se to efficiently inhibit IL-2 production.

It is also of interest that GAMIX alone, at the dose used, does not inhibit ex vivo the proliferation of spleen cells activated by PWM, thus suggesting that gangliosides are immunosuppressive in vivo only in combination with other drugs such as CyA.

In conclusion these findings might suggest a possible use of the association of CyA and GAMIX in immunosuppressive therapy in human transplantation. More information, however, is required on the in vivo renal and hepatotoxicity of this combined therapy, together with new insight into the molecular mechanisms of the immunosuppression exerted by CyA and GAMIX.

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