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Synergism of the malononitrilamides 279 and 715 with cyclosporine A in the induction of long-term cardiac allograft survival

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Abstract We tested here the effects of malononitrilamide (MNA) 279 and MNA 715 (derivatives of A771726, the active metabolite of leflunomide) in monotherapy and in combination with cyclosporine A (CSA) on heterotopically transplanted rat cardiac allografts (BN) [Brown Norway Lewis]. Both MNAs (5–20 mg/kg) displayed a dose-dependent increase of efficacy. The combination of CSA (5 mg/kg) with the MNAs (5 and 10 mg/kg) showed a synergistic effect in the prolongation of allograft survival and in the induction of long-term allograft survival. To investigate the immunological mechanism responsible for long-term allograft survival, we transplanted second set (BN) and third party (Dark Agouti) skin allografts on the tail of long-term surviving rats. The cause for long-term allograft survival turned out to be a donor-specific tolerance. We formulate a hypothesis for the mechanism of the synergism of the combination MNA + CSA in the induction of tolerance.

Key words Malononitrilamides · Cyclosporine A · Transplantation · Tolerance · Immunoregulation

Introduction

The malononitrilamides (MNAs) 279 and 715 are derivatives of A771726, the active metabolite of leflunomide. A771726 and the MNAs 279 and 715 are known to inhibit the de novo biosynthesis of pyrimidines through the blockade of dihydroorotate dehydrogenase (DHODH) [41]. Both MNAs proved to inhibit T-cell and B-cell responsiveness of cells derived from humans and different species [14, 15]. In vivo, the application of MNA 279 and MNA 715 has been effective in the prevention of the development of various murine graft-versus-host diseases and in the prevention of murine systemic lupus erythematosus – like diseases [31, 32, 34]. The MNAs showed efficacy in the suppression of intimal thickening in arterial allografts [20], in the treatment and prevention of acute skin graft rejection reactions [33], and in the control of mouse to rat skin xenograft rejection episodes [29]. Recently, we reported that both MNAs are effective in the prevention and therapeutic treatment of rejection episodes in a rat cardiac transplantation model [16].

In clinical use, immunosuppressive agents are quite often combined to increase the suppression of the immune system and keep the side effects within tolerable ranges. Combinations of an established immunosuppressive agent and a new immunosuppressive drug are used to introduce new drugs for clinical use. Since the already established immunosuppressive agent guarantees a sufficient basic immunosuppression, the efficacy of the new drug can be determined by the additional effects in the therapy of organ transplantation. The combination of two immunosuppressive drugs might result in a synergistic increase of desirable effects. However, the same combination might also show antagonistic effects or an increase of side effects. To minimise the risks of such combination therapies, these should be tested in relevant animal models before their use in humans.

We studied here the effects of the MNAs in combination with cyclosporine A (CSA), which is at present the

clinical standard medication in transplantation. We found here that the MNAs show synergistic effects with CSA in the treatment of cardiac allograft rejection episodes. We demonstrated, furthermore, that the ability of the MNAs to induce donor-specific tolerance by short-term drug administration is enhanced by CSA without an increase of side effects. Finally, we propose a mechanism that might be responsible for the ability of the MNAs in monotherapy or combined with CSA to induce donor-specific tolerance.

Materials and methods

Animals

Male inbred Lewis (LEW) ($RT1^l$) rats served as recipients. Brown Norway rats (BN) ($RT1^n$) (10–14 weeks of age) were used as donors for heterotopic cardiac transplantation. Animals were provided by Charles River (Sulzfeld, Germany). The principles of laboratory animal care were followed.

Immunosuppression

The MNAs 279 and 715 (Hoechst-Werk Kalle-Albert, Wiesbaden, Germany) were dissolved in 1% carboxymethylcellulose and given orally by gavage.

Long-term surviving (LTS) grafts were induced by a 21-day course of MNA 279 or MNA 715, at a daily dosage of 20 mg/kg (per os) or by a combination of the MNAs with 5 mg/kg CSA starting on the day of cardiac transplantation. CSA (Neoral; Sandoz, Basle, Switzerland) was applied orally by gavage from day 0 to day 20.

Cardiac transplantation

For cardiac transplantation the method of Ono and Lindsey [22] was used. The donor rat was anesthetized, the chest opened, and the heart exposed. The venae cavae were ligated, ascending aorta and main pulmonary artery were transected, venae cavae distal to the ligatures were divided, and a ligature was placed around the mass of left atrium and pulmonary veins. Vessels on the lung side of the ligatures were divided and the donor heart was removed. Recipients were anesthetized by ether and received 3 mg/kg Tramadol (Gruenenthal, Germany) for analgesia. The abdomen was opened by a long midline incision and the aorta and inferior vena cava were exposed. The two vessels were separated over a short segment below the renal vessels and end-to-side anastomoses of the aorta and pulmonary artery of the donor heart to the recipient abdominal infrarenal great vessels were performed. Graft function was assessed by daily abdominal palpation. Graft rejection was taken as the complete cessation of heart beats and was confirmed by macroscopic observation upon laparotomy.

Skin transplantation

Donor tail skin was cut into square pieces of 0.5–1 cm^2 and transplanted to the tail of the recipient rats. Rejection was defined as the day when the skin graft was of red-brown color and hard consistency [30].

Results

Dose-response relationship of prophylactically applied MNA 279 and MNA 715. MNA 279 and MNA 715 were applied from day 0 to day 20 at dosages from 2.5 to 20 mg/kg/per day to test their efficacy in the prevention of allograft rejection reactions. A dose-dependent increase of efficacy was seen after the application of MNA 279 and also after the application of MNA 715 (Table 1). Within the 20-mg/kg dosage groups, more than 50% of the grafts survived more than 100 days after discontinuation of any immunosuppressive treatment. CSA was given at a dosage of 10 mg/kg from day 0 to day 20. Allograft survival was prolonged by CSA to 35.4 ± 9.0 days. During CSA treatment, no rejection episodes occurred. All grafts were rejected within 27 days after discontinuation of CSA application and no long-term allograft survival could be seen.

Table 1 Dose-response relationship of prophylactically applied MNA 279 and MNA 715 (*MNA* malononitrilamide, *CSA* cyclosporine A, *LEW* Lewis rat, *BN* Brown Norway rat)

Recipient	Treatment	Donor	Graft	Graft survival (days)	Mean graft survival (days)
LEW	Vehicle only	BN	Heart	4[a], 6, 7, 7, 7, 7, 7, 7, 7, 7, 7	6.9 ± 0.3
LEW	CSA 10 mg/kg	BN	Heart	3[a], 39, 22, 25, 27, 36, 42, 45, 47	35.4 ± 9.0
LEW	MNA 279 2.5 mg/kg	BN	Heart	7, 7, 7, 8, 9	7.6 ± 0.8
LEW	MNA 279 5 mg/kg	BN	Heart	14, 15, 15, 11, 100	31.0 ± 34.5
LEW	MNA 279 10 mg/kg	BN	Heart	4[a], 7[a], > 100, 31, 31, 37	49.8 ± 29.1
LEW	MNA 279 20 mg/kg	BN	Heart	6[a], 7[a], 10[a], 4[a], 11[a], 4[a], 7[a], 32, > 100, > 100, 39, > 100	74.2 ± 31.7
LEW	MNA 715 2.5 mg/kg	BN	Heart	5, 7, 8, 8, 8	7.2 ± 1.2
LEW	MNA 715 5 mg/kg	BN	Heart	4[a], 5[a], 13, 15, 11, 14, 20	14.6 ± 3.0
LEW	MNA 715 10 mg/kg	BN	Heart	6[a], 5[a], 10[a], 7[a], 15, > 100, 20, 33, 17	37.0 ± 32.1
LEW	MNA 715 20 mg/kg	BN	Heart	12[a], 10[a], 8[a], 3[a], 5[a], 6[a], 100, 30, 26, 27, > 100, > 100	63.8 ± 36.2

[a] Animals died with beating cardiac allografts are not included in the determination of median allograft survival

Table 2 Combination of low dose CSA and MNAs

Recipient	Treatment	Donor	Graft	Graft survival (days)	Mean graft survival (days)
LEW	Vehicle only	BN	Heart	6, 7, 5, 7, 8, 6, 9	6.9 ± 1.2
LEW	CSA 5 mg/kg	BN	Heart	0[a], 12, 11, 10, 11	11.0 ± 0.7
LEW	MNA 279 5 mg/kg	BN	Heart	14, 15, 15, 11, > 100	31.0 ± 34.5
LEW	MNA 279 10 mg/kg	BN	Heart	4[a], 7[a], > 100, 31, 31, 37	49.8 ± 29.1
LEW	MNA 279 5 mg/kg CSA	BN	Heart	5 × > 100	> 100
LEW	MNA 279 10 mg/kg CSA	BN	Heart	10[a], 9[a], 5[a], 6[a], 5[a], 5 × > 100	> 100
LEW	MNA 715 5 mg/kg	BN	Heart	4[a], 5[a], 13, 15, 11, 14, 20	14.6 ± 3.0
LEW	MNA 715 10 mg/kg	BN	Heart	6[a], 5[a], 10[a], 7[a], 15, > 100, 20, 33, 17	37.0 ± 32.1
LEW	MNA 715 5 mg/kg CSA	BN	Heart	27, 34, 47, 10, > 100	43.6 ± 30.6
LEW	MNA 715 10 mg/kg CSA	BN	Heart	5 × > 100	> 100

[a] Animals died with beating cardiac allografts are not included in the determination of median allograft survival

Table 3 Demonstration of donor-specific tolerance in animals treated with MNA monotherapy and combinations of MNAs with CSA (*DA* Dark Agouti rat)

Recipient	Donor	Graft	Graft survival (days)	Mean graft survival (days)
LEW	BN	Skin	7, 8, 7, 8, 9	7.5 ± 0.7
	DA	Skin	8, 7, 8, 9, 10	8.0 ± 1.0
LTS (279) LEW	BN	Skin	> 30[b], > 30[b], > 30[b], > 30[b], > 30[b]	> 30
	DA	Skin	9, 10, 10, 8, 11	9.3 ± 1.0
	BN[a]	Heart	> 50, > 50	> 50
LTS (715) LEW	BN	Skin	> 30[b], > 30[b], > 30[b], > 30[b], > 30[b]	> 30
	DA	Skin	9, 8, 10, 8, 8	8.8 ± 0.8
	BN[a]	Heart	> 50, > 50	> 50
LTS (279 + CSA) LEW	BN	Skin	5 × > 30[b]	> 30
	DA	Skin	8, 9, 10, 8, 11	8.8 ± 1.2
	BN[a]	Heart	> 50, > 50, > 50	> 50
LTS (715 + CSA) LEW	BN	Skin	5 × > 30[b]	> 30
	DA	Skin	7, 8, 10, 10, 8	8.8 ± 1.2
	BN[a]	Heart	> 50, > 50	> 50
LEW	BN	Skin	4, 5, 3, 3, 4	4.0 ± 3.8
BN sensitized	DA	Skin	10, 9, 11, 12, 8	10.5 ± 1.4
LEW	BN	Skin	8, 12, 8, 11, 8	9.8 ± 1.7
MNA 279 pretreated	DA	Skin	10, 11, 10, 9, 9	10.0 ± 0.7
LEW	BN	Skin	9, 9, 9, 10, 9	9.3 ± 0.4
MNA 715 pretreated	DA	Skin	9, 10, 9, 9, 8	9.3 ± 0.6
LEW	BN	Skin	9, 7, 9, 8, 7	11.0 ± 0.9
CSA pretreated	DA	Skin	8, 10, 7, 9, 8	11.4 ± 1.0

[a] The BN hearts were transplanted into long-term survival animals that received MNA or MNA + CSA before a second set (BN) and third party (DA) skin transplant

[b] Second set skin transplant thin and soft, complete depigmentation of the skin but not of the hair

Synergism of MNA + CSA combination therapy

Cardiac allograft survival turned out to be longer after application of CSA at a dosage of 5 mg/kg (11.0 ± 0.7 days against 6.9 ± 1.2 days in the control group). However, all cardiac allografts were rejected on CSA therapy at this dosage (Table 2). Rejection reactions were observed during treatment with MNA 279 at a dosage of 5 mg/kg. On the same monotherapy at a dosage of 10 mg/kg, no rejection episodes occurred. If the subtherapeutic dosage of 5 mg/kg CSA was combined with 5 or 10 mg/kg MNA 279, no rejection episodes were observed during treatment and even after discontinuation of any immunosuppressive treatment. If MNA 715 was combined with 5 mg/kg CSA, a significant prolongation of allograft survival was seen in the 5-mg/kg MNA dosage group. In this same group, we also observed in one case the induction of long-term al-

lograft survival. In the MNA 715 10-mg/kg dosage group, the combination with a suboptimal dosage of 5 mg/kg CSA resulted in induction of long-term allograft survival in all animals (Table 2).

Demonstration of donor-specific tolerance in animals with LTS cardiac allografts

To investigate the mechanism of cardiac acceptance in tolerant animals, second set (BN) and third party [Dark Agouti (DA)] skin transplantations were performed on animals that had accepted their grafts for more than 100 days subsequent to discontinuation of any immunosuppressive treatment. Naive (not tolerant) animals rejected DA skin grafts within 8.0 ± 1.0 days and BN skin grafts within 7.5 ± 0.7 days. LEW rats that were tolerant to BN cardiac allografts subsequent to MNA 279 [LTS (279) LEW] treatment rejected third party DA skin grafts after 9.3 ± 1.0 days. In contrast, the same animals did not reject second set BN skin grafts during the same observation period of 30 days. LEW rats that were tolerant to BN cardiac allografts subsequent to MNA 715 [LTS (715) LEW] treatment rejected third party DA skin grafts at 8.8 ± 0.8 days but tolerated BN second set skin grafts. Rats that were tolerant to BN cardiac allografts subsequent to the combination therapy (MNAs + CSA) tolerated second set BN skin grafts and rejected DA third party skin grafts (Table 3).

Although the second set BN skin grafts in LTS (279) LEW, LTS (279 + CSA) LEW, LTS (715) LEW, and in LTS (715 + CSA) LEW animals were not rejected during the observation period (> 30 days), some changes of the skin allografts occurred. Between day 10 and day 25 after the skin transplantation, a hyperkeratosis of the second set (BN) skin allografts was observed. At day 30, the second set (BN) skin transplants were soft, thin, fragile, and completely depigmented. The hair of the second set (BN) skin allografts was still pigmented and viable (Table 3). After the second set (BN) skin transplants had been observed for at least 30 days, we tried to transplant a second BN heart into the abdomen of these long-term surviving animals. Neither the animals that were made tolerant by MNA monotherapy, nor the animals rendered tolerant by the combination therapy (MNA + CSA) displayed a rejection episode against the second intra-abdominal BN heart allograft within the observation period (50 days; Table 3).

Discussion

MNA monotherapy given as prophylactic treatment

MNA 279 and MNA 715 in monotherapy were tested for their efficacy in the prevention of cardiac allograft rejection episodes at dosages ranging from 2.5 to 20 mg/kg. A dosage of 2.5 mg/kg of either substance was not effective. Both MNAs showed a dose-dependent increase of efficacy starting from 5 mg/kg. There was also a dose-dependent increase of the induction of long-term allograft survival (up to 60% in the highest dosage groups). Animals with LTS allografts did not reject the allografts even after discontinuation of any immunosuppressive treatment (observed for up to 200 days).

Combination of CSA with the MNAs

When CSA was combined with either MNA, allografts survived longer than when CSA, MNA 279 or MNA 715 were given in monotherapy. Since the survival prolongation observed on CSA + MNA combination therapy was even longer than the simple addition of the prolongation obtained with the monotherapies, we conclude that CSA and MNA have a synergistic action in the prolongation of cardiac allograft survival. Moreover, this synergistic action of the combination therapy was also seen in the induction of long-term allograft survival.

Second set (BN) and third party (DA) skin transplantation

In this experiment, we transplanted skin allografts to naive LEW rats and to LTS LEW rats that had accepted a heart allograft even after discontinuation of any immunosuppressive therapy. The naive rats, BN presensitized, MNA 279 pretreated, and MNA 715 pretreated rats rejected both skin allografts. The LTS rats accepted the skin allograft derived from the same rat strain (BN) from which they had already accepted the heart but rejected the skin allograft derived from another rat strain (DA). Although the BN skin allografts were not rejected by the LTS rats, the structure of the skin changed and displayed a hyperkeratosis and depigmentation. These changes in BN skin allografts raised the question whether this hyperkeratosis was the result of a delayed rejection of the BN skin transplant. Since the LTS animals did not reject the second BN cardiac allograft we had transplanted into their abdomen, we conclude that the LTS animals have developed a donor-specific tolerance. The donor-specific tolerance was equally induced by MNA 279, MNA 715 in monotherapy, and CSA +

MNA combination therapies. However, the combination therapies were more effective than the monotherapies.

A cause for this donor-specific tolerance could be the selective elimination of all alloreactive cells of the immune system by the application of the MNAs. Another mechanism for the induction of tolerance by the MNAs could be the induction of a regulatory immune response that protects the graft from destruction. At present, we favor the hypothesis that a regulatory immune response is the cause for the observed tolerance. Our opinion is supported by the fact that the tolerance was stable for more than 200 days. This period was long enough to allow new alloreactive cells to emerge from the bone marrow and to reject the allograft. Therefore, the prolongation of the allograft survival for such a long time can only be explained by a regulatory immune response. However, since we cannot completely exclude the possibility that the thymus or cells of the bone marrow were damaged by the short-term MNA administration, the tolerance could also have been the result of an inhibition of the forming or maturation of new alloreactive cells. Further investigations must be performed in order to better understand the mechanism that underlies the observed donor-specific tolerance.

Possible mechanisms for the synergism of the combination therapy MNAs + CSA, in the induction of tolerance

We review here features of the immunoregulatory immune responses that have already been reported in the literature in order to find out their common characteristics.

Interleukin-10 and transforming growth factor (TGF)-β1 are cytokines that are produced by T-cells; these cytokines are able to prolong allograft survival [24–26]. In addition, TGF-β1 promotes the outgrowth of immunoregulatory T-cell populations [27]. Antigen presentation is an important step in the initiation of immune responses and can be inhibited or reduced by interleukin-10 and TGF-β1 [26, 40]. The development of immunoregulatory T-cell populations can be induced by low-dose antigen feeding [4]. Suboptimal antigenic peptides can induce regulatory T-cell populations that produce the immunosupressive cytokines interleukin-10 or TGF-β1 [18, 37, 42]. The metabolic activity of these T-cells, measured by the incorporation of thymidine, is reduced [42]. Some immunoregulatory T-cell populations produce soluble T-cell receptors that are reliable for the antigen-specific immunosuppressive activities of these cells [8, 9]. The reduction of T-cell receptors on the surface has also been demonstrated to be a feature of cells involved in immunosuppressive regulatory immune responses [28]. Some of these immunosuppressive acting regulatory T-cells display a reduced Ca^{++} flux in response to a mitogen stimulation and they also show changes/disturbances in the signal transduction pathways downstream of the T-cell receptor [2, 3, 38]. Ca^{++} influx and the activation of signal transduction pathways are common signs of T-cell activation.

All these regulatory immune responses turn out to inhibit or reduce the antigen presentation or recognition process of the T-cells. Therefore, a common feature found in these regulatory immune responses is the reduced activation of the immunoregulatory "protective" T-cells. Thus we formulate the hypothesis that the allograft rejection-mediating or "effector" T-cells are more activated than the "protective" T-cells in the process of T-cell-dependent immune reactions. It is well known in pharmacology that highly activated, metabolically active cells respond better to cytostatic/cytotoxic drugs than metabolically inactive cells. This offers the chance to establish a regulatory immune response by eliminating the metabolically highly activated effector T-cells by the application of cytostatic/cytotoxic drugs. One should bear in mind, however, that not all types of cytostatic/cytotoxic drugs induce a regulatory immune response because activated T-cells display protective mechanisms against some of these cytostatic/cytotoxic agents [6, 10, 11, 17, 21, 39]. However, MNAs do not to belong to this type of drug. As a matter of fact, it has already been demonstrated that the MNAs and the mother compound of the MNAs, the active metabolite of leflunomide, A771726, inhibit de novo pyrimidine synthesis by blocking the enzyme DHODH [41]. This inhibition affects preferentially activated cells of the immune system [1, 5, 7, 19, 23, 36]. Since the pyrimidine salvage pathways support resting cells of the immune system with sufficient amounts of pyrimidines, the resting cells are not sensitive to a blockade of de novo pyrimidine synthesis [7, 19].

As a result of our first hypothesis (according to which protective T-cells are less activated than the effector T-cells) and of the fact that MNAs preferentially inhibit highly activated cells of the immune system, we propose the following mechanism that could possibly explain the tolerance-inducing effect of the MNAs.

MNAs induce tolerance by preferentially inhibiting the effector T-cells and by sparing the protective T-cells. This would then allow the formation of an immune microenvironment that maintains the tolerance even after the MNA application has been stopped. CSA inhibits interleukin-2 production and also increases the production of the immunosuppressive cytokine TGF-β1 [12, 13, 35]. In vitro experiments showed the ability of TGF-β1 to induce protective regulatory immune functions in T-cells, which then begin themselves to produce protective immunoregulatory cytokines such as interleukin-10 and TGF-β1 [27]. The result is a self-enhancing circuit in which TGF-β1 induces the forming of reg-

ulatory protective T-cells and where the latter produce TGF-β1 and vice versa. CSA plays a role in the formation of the protective T-cells via TGF-β1 and also inhibits the production of the immunostimulatory cytokine interleukin-2, which stimulates the effector T-cells. Our hypothesis, according to which, MNAs, on the one hand, spare the protective T-cells and, on the other hand, inhibit the effector T-cells, would then explain the synergism of CSA and MNAs in our experiments.

Additional experiments are, however, required to further support these possible mechanisms for the synergism of the combination therapy (CSA + MNAs) in the induction of tolerance. First of all, one should find out if an inhibition of the tolerance induction appears after antagonization of TGF-β1 effects. Secondly, it should be demonstrated that the tolerance-maintaining cells are involved in the impaired antigen recognition system. And thirdly, one should prove the synergistic effects of the MNAs in inducing tolerance with TGF-β1 or substances that increase TGF-β1 effects, such as FK506 for example.

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