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Georg A. Böhmig
Thomas Wekerle
Marcus D. Säemann
Josef Kovarik
Gerhard J. Zlabinger

Induction of alloantigen-specific hyporesponsiveness in vitro by n-butyrate: antagonistic effect of cyclosporin A

G. A. Böhmig · M. D. Säemann · G. J. Zlabinger (✉)
Institute of Immunology,
University of Vienna, Borschkegasse 8A,
A-1090 Vienna, Austria
Tel. +431 401 54/356;
Fax +431 4086670

T. Wekerle
Department of Transplant Surgery,
University of Vienna,
Währinger Gürtel 18–20,
A-1090 Vienna, Austria

J. Kovarik
Department of Internal Medicine,
University of Vienna,
Währinger Gürtel 18–20,
A-1090 Vienna, Austria

Abstract The short-chain fatty acid n-butyrate has recently been shown in vitro to specifically downregulate T cell reactivity to nominal antigen or to alloantigen, which possibly results from inhibition of cell cycle progression in early G_1 phase during antigen contact. In the present study, we investigated the effect of cyclosporin A (CyA) on the modulation of alloreactivity in human mixed lymphocyte culture (MLC) by n-butyrate. Whereas in primary culture, CyA additively enhanced inhibition of DNA synthesis by n-butyrate, the effect of this agent on secondary T cell reactivity was clearly antagonized by CyA. Thus, specific downregulation of proliferative responsiveness to restimulation with antigen from the original donor, observed in cultures pretreated with n-butyrate alone, was at least partially prevented by the addition of CyA to the primary culture. Our in vitro finding indicates that specific downregulation of T cell alloreactivity by n-butyrate might depend on a calcium-dependent T cell receptor (TCR)-mediated signal sensitive to the immunosuppressive action of CyA.

Key words n-Butyrate · Cyclosporin A · T cell · Alloantigen · Hyporesponsiveness

Introduction

n-Butyrate has been described as having multiple biological effects on various types of mammalian cells, such as modulation of gene expression, inhibition of proliferation, and induction of cellular differentiation [1]. This short-chain fatty acid, as well as related organic compounds, has also been reported to block proliferative responses of murine or human lymphocytes to mitogens [2–4], and to suppress the proliferation as well as generation of cytotoxic T cells in human primary and secondary MLC [5]. Furthermore, supplementation of drinking water with n-butyric acid resulted in modest but significant prolongation of skin allograft survival in mice [5]. Recently, Gilbert and Weigle [6] demonstrated in a human HGG-specific T cell clone that antigen contact in the presence of n-butyrate results in a state of antigen-specific unresponsiveness. Blockade of cell cycle progression in G_{1a} phase during antigen contact was proposed to be the mechanism underlying anergy induction in this model [6, 7]. We could further demonstrate that beyond inhibiting T cell proliferation in primary MLC, pretreatment of primary cultures with n-butyrate also resulted in a profound state of alloantigen-specific hyporesponsiveness as assessed in restimulation culture, suggesting a possible application of this agent for tolerance induction to allografts [8].

A series of data indicates that the immunosuppressive drug CyA might alter distinct forms of immunological tolerance [9]. Thus, CyA has been shown to prevent the induction of T cell anergy in vitro [10–12] as well as in vivo [13, 14]. Furthermore, this agent might interfere with the process of clonal deletion during thymic maturation [15–17]. On the other hand, however, CyA is well known to induce tolerance to allografts particularly in rodents [18]. In addition, this immunosuppressant has recently been shown to enhance peripheral T cell deletion [13, 14] and to induce alloantigen-specific anergy

in vitro when combined with a monoclonal antibody against B7 [19].

The present study was designed to examine the impact of CyA on the modulation of alloreactivity by n-butyrate. Our observation that CyA enhances the inhibitory effect of n-butyrate on DNA synthesis in primary MLC, but antagonizes specific downregulation of T cell reactivity as assessed in secondary culture, suggests that the induction of specific hyporesponsiveness by n-butyrate critically depends on a calcium-dependent TCR-triggered signal that is sensitive to the immunosuppressive action of CyA.

Materials and methods

Primary MLC

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 10 % fetal calf serum that had been inactivated at 56 °C for 30 min. For the primary culture, 4×10^4 PBMC from healthy volunteers were mixed with 4×10^4 irradiated (6000 rad) PBMC from unrelated donors in medium with or without n-butyrate (Sigma Chemical Co., St. Louis, Mo.) or CyA (a gift from Dr. Wiskott, Sandoz Ltd.). The cultures were set up in triplicate at 37 °C in a 5 % CO_2 atmosphere in round-bottomed 96-well plates. Proliferation was assessed on day 7 of culture. For measurement of DNA synthesis, the cultures were pulsed with 1 μCi [^{3}H]thymidine 16 h prior to harvesting. Incorporated radioactivity was measured by liquid scintillation spectrometry. Data are reported as mean cpm ± SD of triplicate cultures.

Secondary MLC

For secondary MLC, 1×10^6 PBMC were cocultured with an equal number of irradiated allogeneic PBMC in 75 × 12 mm tissue culture tubes in the presence or absence of the above-mentioned agents using culture conditions as described for primary MLC. After 7 days of culture, the cells were washed and incubated in fresh medium for another 7 days. Precultured cells (3×10^3) were then rechallenged with an equal number of freshly isolated irradiated autologous cells, of irradiated cells from the original donor or from unrelated third party donors, or with phytohemagglutinin (PHA) (1 μg/ml; Murex Diagnostics Limited, Dartford, UK). Secondary cultures were carried out in round-bottomed 96-well plates, and DNA synthesis was assessed on four consecutive days (days 3–6).

Flow Cytometry

For the determination of cell viability, cells were harvested at the day of restimulation, pelleted, and resuspended in PBS containing proidium iodide (PI) (0.1 μg/ml). After 15 min incubation at room temperature, the percentage of viable cells was assessed according to scatter characteristics and exclusion of PI using a FACScan (Becton Dickinson, Sunnyvale, Ca).

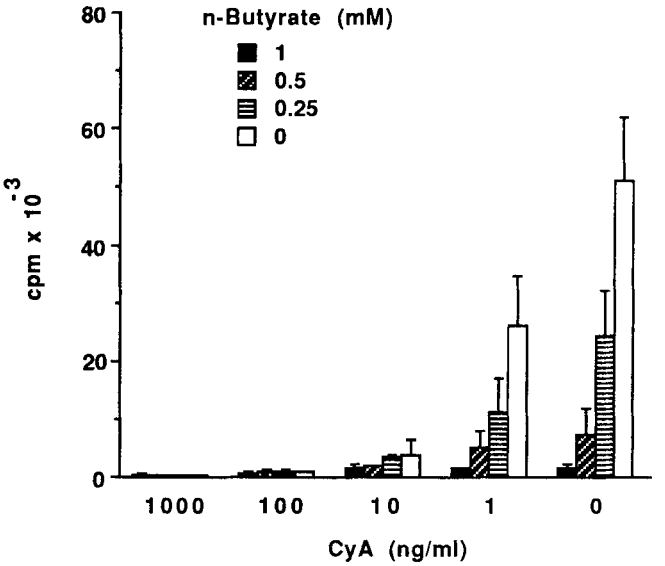

Fig. 1 Effect of CyA on inhibition of T cell alloreactivity in primary MLC by n-butyrate. DNA synthesis was assessed on day 7 of culture. Mean cpm ± SD of triplicate cultures are depicted. The experiment shown is a representative of two experiments each testing two different donor combinations

Results

Effect of CyA on inhibition of primary alloresponses by n-butyrate

In order to examine the effect of CyA on n-butyrate-mediated suppression of DNA synthesis in primary MLC, freshly isolated PBMC were mixed with irradiated allogeneic PBMC in medium containing n-butyrate at increasing concentrations in the presence or absence of CyA at various concentrations. As shown in Fig. 1, the n-butyrate dose dependently inhibited proliferation, with complete inhibition at 1 mM. CyA additively suppressed proliferative alloresponsiveness in n-butyrate-treated cultures.

Effect of CyA on specific downregulation of proliferative T cell alloreactivity by n-butyrate

To investigate the impact of CyA on the induction of alloantigen-specific hyporesponsiveness by n-butyrate, primary cultures were carried out in medium containing this agent at 1 mM in the presence or absence of CyA. CyA was added at 1 μg/ml, a concentration which in all experiments blocked primary alloresponses by more than 95 % (Table 1). In order to determine proliferative T cell responsiveness in restimulation culture, cells from primary cultures were washed on day 7 and incubated for another week in fresh medium until they were restim-

Table 1 Effect of CyA on the induction of alloantigen-specific hyporesponsiveness by n-butyrate. The results obtained in two different donor combinations *(A, B)* are depicted. Similar data were obtained in another two responder-stimulator combinations. Data are reported as mean cpm ± SD

Additions to primary MLC	Primary culture (day 7)	Percentage viability	Secondary culture (peak proliferation)			
			Autologous antigen	Specific antigen	Third party antigen	Phytohemagglutinin
A						
None	69254 ± 11326	46	3177 ± 1016	71632 ± 7035	30712 ± 2901	51580 ± 3072
n-Butyrate	1100 ± 413	42	2104 ± 1077	10690 ± 1330	24258 ± 4952	56412 ± 2518
CyA	665 ± 207	45	1016 ± 651	17928 ± 1296	22781 ± 10624	68481 ± 5844
CyA + n-butyrate	285 ± 147	44	3710 ± 1729	29956 ± 5371	47535 ± 7106	52734 ± 3650
B						
None	43095 ± 8695	42	1575 ± 756	20858 ± 5194	13673 ± 3641	38425 ± 3370
n-Butyrate	1975 ± 405	48	1597 ± 386	12041 ± 4262	12987 ± 4181	67952 ± 1150
CyA	561 ± 214	51	967 ± 706	23965 ± 4241	14204 ± 478	51499 ± 655
CyA + n-butyrate	238 ± 39	51	2589 ± 1209	39585 ± 1649	10910 ± 5215	51008 ± 7213

ulated. To exclude non-specific toxic effects of the tested agents, the cells were harvested after this culture period and cell viability was assessed by PI staining. No significant reduction of cell viability was observed in cultures pretreated with n-butyrate, CyA, or with a combination of both substances (Table 1). Next, pretreated cells were rechallenged with freshly isolated irradiated autologous cells, irradiated cells from the original donor, from unrelated third party donors, or with PHA (1 μg/ml). In Table 1, the results obtained in two representative responder-stimulator combinations are shown. As previously reported [8], pretreatment of primary MLC with n-butyrate resulted in a marked reduction of proliferative reactivity to alloantigen from the original donor, whereas no such effect was observed regarding responsiveness to third party antigen or to mitogenic stimulation. Compared to cultures pretreated with n-butyrate alone, the addition of CyA to n-butyrate-treated primary cultures resulted in a marked, approximately three-fold, increase of alloresponsiveness to the same donor in secondary culture. In cultures pretreated with both CyA and n-butyrate, proliferative responses to the specific donor were clearly delayed (peaking on day 5 or 6) when compared to responses observed in control cultures or cultures pretreated with n-butyrate alone (peaking on day 3 or 4) and, thus, were characteristic of primary type alloresponses (not shown). Accordingly, as observed in three out of four donor combinations, in cultures pretreated with both CyA and n-butyrate, specific responses did not achieve levels observed in primed control cultures. Furthermore, in three donor combinations, the addition of CyA to primary MLC resulted in a significant decrease of reactivity to the specific donor, whereas reactivity to third party antigen or to PHA was not or only slightly reduced (see also Table 1). After pretreatment with both CyA and n-butyrate, responsiveness to the original donor was increased above levels observed in cultures pretreated with CyA alone.

Discussion

We have previously shown that beyond inhibition of primary alloresponses, the short-chain fatty acid n-butyrate induces a state of alloantigen-specific hyporesponsiveness, when added during primary contact with alloantigen [8]. In the present study, we analyzed the effect of the immunosuppressant CyA on n-butyrate-mediated inhibition of DNA synthesis in primary MLC and, further, on the induction of donor-specific hyporesponsiveness. Here, we describe that this agent enhances suppression of proliferation in primary MLC, but clearly antagonizes specific downregulation of T cell reactivity by n-butyrate. The latter finding suggests that specific downregulation of T cell alloreactivity might depend on an active TCR-triggered intracellular signal that is sensitive to the immunosuppressive action of CyA. Our finding that in most cases CyA did not restore specific T cell reactivity to levels observed in primed control cultures might be explained by prevention of T cell priming to alloantigen in cultures pretreated with both CyA and n-butyrate, as supported also by the time kinetics of proliferative responses to antigen from the original donor, which were delayed when compared to the secondary type response in untreated cultures.

CyA has been shown to inhibit a calcium-dependent TCR signal transduction pathway by blocking the activity of calcineurin, a calcium- and calmodulin-dependent protein phosphatase, which is responsible for the dephosphorylation and nuclear translocation of the cytoplasmic subunit of NF-AT (nuclear factor of activated T cells). As a consequence, the expression of numerous T cell activation genes, including genes encoding cytokines, is blocked [20].

Besides its immunosuppressive properties, CyA has been shown to alter distinct forms of immunological tolerance. It has also been reported that CyA prevents the induction of anergy in vitro [10–12] as well as in vivo

[13, 14], indicating an important role of TCR-triggered calcium-dependent signal transduction in the induction of functional T cell unresponsiveness. Recently, CyA has been shown to inhibit the induction of binding activity to the negative regulatory element A (NRE-A) of the IL-2 promotor following anergy induction in a human T cell clone [21]. These data are in line with the previously suggested model, that the induction of clonal anergy might result from the accumulation of a TCR-triggered negative regulator of IL-2 gene transcription, that is normally diluted out by proliferation [22, 23]. In addition, CyA has also been reported to interfere with other tolerance mechanisms, e.g., the blocking of intrathymic clonal deletion [15–17] or the inhibition of suppressor cell mechanisms [9].

In contrast to its reported prohibitive effects on various tolerance mechanisms, a short course of CyA has been reported to induce by itself a state of long-term tolerance to allografts in rats and in other species, which was attributed to the action of suppressor cells [18]. Furthermore, CyA has also been shown to increase peripheral deletion of reactive T cells induced by superantigens or anti-TCR monoclonal antibody in mice [13, 14]. Recently, Van Gool et al. [19] demonstrated that CyA synergizes with blockade of costimulation using a monoclonal antibody to B 7–1 in the induction of alloantigen-specific anergy in freshly isolated human T cells as assessed at the cytotoxic effector level. These authors suggested that, in this model, CyA might block additional calcium-dependent costimulatory signals and postulated that a calcium-independent limb of the TCR signaling pathway is required for anergy induction and remains unaffected by their treatment protocol [19].

Pretreatment of primary cultures with CyA alone sometimes led to a significant reduction of T cell responsiveness to the specific donor in secondary MLC, which appeared not to be a result of non-specific toxicity. This observation might fit in with a previous report demonstrating specific downregulation of alloantigen-specific T cell reactivity by CyA in murine MLC [24]. In addition, in human MLC, alloantigen-activated suppressor cells have been reported to be more resistant to CyA treatment than effector cells [25, 26]. Interestingly, the combined addition of both CyA and n-butyrate increased specific reactivity above levels observed in cultures pretreated with CyA or n-butyrate alone, suggesting that inversely n-butyrate might interfere with the modulation of T cell alloreactivity by CyA.

In conclusion, the observation that CyA antagonizes the induction of alloantigen-specific hyporesponsiveness by n-butyrate indicates that specific downregulation of T cell alloresponsiveness by n-butyrate critically depends on a calcium-dependent TCR-triggered signal that is sensitive to the immunosuppressive action of CyA. Our finding further supports previous results showing that immunosuppression by CyA might paradoxically interfere with distinct strategies of tolerance induction, which might be of importance in the introduction of new therapeutic strategies to induce tolerance to allografts.

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