The Effect of Mafosfamide on Differential Activation of T-helper Subsets

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Summary

The differential activation of Th1 and Th2 cells using mafosfamide (Mafo) was investigated *in vitro* in a rat model of alloreactivity. The results of this study are compatible with the hypothesis that Mafo, like its parent compound cyclophosphamide, is selectively active against Th2-type T-helper cells when administered at low doses (<0.1μM). Mafo suppressed the activation of suppressor cells and increased cytotoxicity and proliferation of CD4 rather than CD8 T-cells. Interestingly, treatment with Mafo reversed the inhibitory effect of cAMP on lymph node cell proliferation. It seems to interfere with second messenger signalling pathways, through interactions in a subtle way with the cAMP-dependent protein kinase system. Therefore, Mafo can open up new possibilities for examination in the colonogenic cell assay, *in vitro*, and also for the regional therapy of allergy, specifically for low doses of Mafo, as an immunomodulator.

Introduction

It has been known for some time that treatment of animals with low doses of cyclophosphamide (CYP) could augment cell-mediated immune responses and overcome suppression (Ben-Efrain 2001, Matar *et al* 2002). We have recently

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shown that CYP at low doses (<25mg/kg) accelerates kidney allograft rejection *in vivo*, and cells from CYP-treated rats give higher MLR responses, and generate more cytotoxic T-cells, but fails to show any suppressive activity (Asli *et al* 2004). In addition, cells from CYP-pretreated animals produce abundant IL-2 and IFN-γ, but do not produce any IL-5 (Matar *et al* 2002). This finding is strongly supporting our hypothesis that CYP selectively inactivates Th2-type T-helper cells. To enable us to pursue these studies we needed to treat lymphocytes *in vitro* with the agent. CYP is not biologically active *in vitro* without metabolic conversion and cannot be used to treat lymphoid cells, but mafosfamide (Mafo) is an analogue of CYP that is active *in vitro* (Boal *et al* 1994). Mafo has been shown to induce inhibition of histone acetylation as demonstrated for tumour cells (Grunicke *et al* 1983). It is also a cytotoxic compound active both *in vitro* and *in vivo*, and is highly effective *in vitro* against rodent tumour cell lines at a concentration of 1 to 10 μg/ml (Atassi *et al* 1984). It has been reported that Mafo is almost twice as active as CYP (Klein *et al* 1984) at low doses, could enhance host anti-tumour immunity in animals (Reissmann *et al* 1989) and IL-2 activity (Hassan 1994).

The aim of this study was to investigate the differential activation of Th1 and Th2 cells using Mafo, in a rat model of alloreactivity in order to introduce it instead of CYP in cell-mediated immune response studies as an immunomodulator.

Materials and Methods

Animal. Male rats aged 8-12 weeks of inbred strains DA (Rt1^a) and PVG. Rt1^{u}7b were used.

Preparation of cell suspension. Rat lymph nodes (axillary, cervical and mesentric) were aseptically removed and disaggregated with Hanks Balanced Salt Solution (HBSS). The cells were washed twice with HBSS. Viable nucleated cells were counted using trypan blue staining and adjusted to 5×10^6 cells/ml with complete medium (RPMI-1640 with glutamine (GIBCO) and supplemented with

7% fetal calf serum, 5µM 2-Mercaptoethanol, 0.45mg/ml streptomycin, 0.45mg/ml penicillin G, and 0.9mg/ml kanamycin).

Fractionation of T-cells. T-cell populations were separated into $CD4^+$ and $CD8^+$ T-cell subpopulations as described elsewhere by Asli *et al* (2004) using immuno magnetic particles (Metachem Diagnostics Ltd UK). The purity of the isolated subpopulation was always >90% as assessed by using a FACScan (Becton Dickinson) flow cytometry.

Treatment of cells with Mafo. Mafo was obtained from ASTAMedica AG (Frankfurt, Germany). It was reconstituted in culture medium immediately before use. Mafo was added at graded concentrations (10-100nM) to the fractionated or unfractionated responder (PVG.RT1^{u}7b) cell suspensions, which were at 5×10^6 cells/ml in complete medium. The Mafo-treated cells were incubated at 37°C for 20 min then washed and resuspended in complete culture medium, counted and adjusted to the required concentrations for micro MLR (at 1×10^7 cells/ml) and bulk MLR (at 5×10^6 cells/ml).

Mixed lymphocyte reaction (MLR). Mafo-treated and untreated (normal) responder cells from PVG.RT1^{u}7b rats, and irradiated stimulator cells from DA rats at a concentration of 1×10^7 cells/ml were prepared. Stimulator cells were activated by exposure to 2000 rad irradited at 180 rad/min from a ^{37}Ci source. All assays were performed using a 1:1 stimulator to responder cell ratio in triplicate in 96 well flat bottomed microtitre plates with 200µl per well, and were incubated at 37°C with 5%CO_2 in a humidified incubator. On day 4 of culture, 1µ Ci of ^{3}H-thymidine (Amersham) was added to each well. On day 5 the cells were harvested on to a glass fiber filter paper and incorporated ^{3}H-thymidine was measured using a β-counter.

Cytotoxicity and suppressor assays. A bulk mixed lymphocyte cultures was set up for the cytotoxicity and suppressor assays by mixing 10ml aliquots of each cell suspension type (responders and stimulators as above) in a 1:1 ratio into 25ml culture flasks and the cells were incubated for 4-6 days at 37°C with 5%CO_2 prior

to use. Cytotoxicity assay was a 4h ^{51}Cr-release assay, based on the method of Brunner *et al* (1968). For suppressor assay, viable lymph node cells from day 5 bulk MLR cultures were separated on a ficoll-hypaque gradient and resuspended at 5×10^{-6}cells/ml in complete culture medium. They were then irradiated and added as modulator cells to a second fresh MLR performed in 96 well plates using responder (PVG.RT1^{u}7b), stimulator (irradiated DA) and modulator (irradiated PVG.RT1^{u}7b anti DA) cells in a 1:1 ratio. Triplicate MLR cultures were set up in flat-bottomed microtitre plates. All cells were added in 50μl volumes (Responder, Stimulator and Modulator cells) and the final well volumes were adjusted to 200μl with extra culture medium. Plates were then incubated and proliferation was quantitated by the incorporation of ^{3}H-thymidine added on day 4 of culture.

Statistical analysis. Results are expressed as mean±standard deviation (SD) of triplicate samples. The significance of the differences between means was evaluated by the Student's *t*-test.

Results

Effect of pretreatment of responder cells with Mafo on proliferation in the MLR. The proliferative response of Mafo-pretreated responder cells *in vitro* was dependent on the dose of Mafo added to the culture. Low doses (10-100nM) of Mafo caused a significant increased proliferation, but doses higher than 1μM caused a significant decrease in proliferation (Figure 1).

Effect of pretreatment of responder cells with Mafo on the generation of cytotoxic T-cells. Pretreatment of responder cells i*n vitro* with 0.1 to 1.0μM Mafo increased the generation of specific cytotoxic T-cells in subsequent allogeneic MLR (Figure 2). A higher concentration of Mafo (1.0mM) abolished the generation of cytotoxicity.

Effect of pretreatment of responder cells with Mafo on the generation of suppressor T-cells. The effect of pretreatment of responder cells with Mafo was to

establish the activation of suppressor cells (figure not shown). Instead, in the suppressor assay, modulator cells pretreated with from 0.1µM Mafo caused an increase in proliferation.

Figure 1: *Effect of addition of Mafo in vitro on the proliferative response of rat lymph node cells in the allogeneic mixed lymphocyte reaction (MLR). Data expressed as mean ^{3}H-thymidine uptake±S.D (% of control, n=4 experiments). *P <0.05 (paired Student's t-test)*

Figure 2. The *effect of addition of Mafo in vitro on the activation of cytotoxic T-cells from rat lymph node cells in the allogeneic mixed lymphocyte reaction (MLR). Cells (effectors) from day 5 MLR, activated in continuous presence of Mafo or RPMI-1640 (control), incubated with ^{51}Cr-labeled targets at effector: target ratio shown in a 4 hrs ^{51}Cr-release assay. Data expressed as mean % lysis±SD of triplicates were shown*

Effect of pretreatment of CD4 and CD8 T-cells with Mafo on their proliferation in MLR. There was significantly increased proliferation in MLR when the CD4 T-cells had been pretreated with 0.1µM Mafo (Figure 3). CD8 T-cells were not significantly affected by this dose of Mafo. Higher doses, 10 and 100µM Mafo, were suppressive of both CD4 and CD8 cells.

Figure 3. *The effect of in vitro treatment of CD4 and CD8 lymph node cells, separately, with Mafo on the MLR.. Proliferation was quantitated by incorporation of ^{3}H-thymidine at day 4 of culture. The results of three independent experiments were combined and are presented as mean ^{3}H-Thymidine uptake±S.D (% of fractionated control MLR proliferation, n=3 experiments). *P<0.05 compared with normal CD4+ normal CD8 MLR culture*

Effect of pretreatment of CD4 and CD8 T-cells with Mafo on the generation of cytotoxic function. Normal, untreated CD4 or CD8 T-cells on their own did not generate significant cytotoxic activity in MLR, while the mixture of untreated CD4 and CD8 cells did lead to the generation of specific cytotoxic cells. When CD4 T-cells, pretreated with 10nM-0.1μM Mafo were mixed with normal CD8 T-cells there was an increase in generation of specific cytotoxicity (Table 1).

Table 1. *The effect of addition of Mafo in vitro on the activation of cytotoxic T-cells from rat lymph node cells in the allogeneic mixed lymphocyte reaction*

Fractionated T-cells Dose of Mafo 0.1μM	Cytotoxic activity of lymph node cells % of specific lysis of ^{51}Cr-labelled target	
	50:1 (E:T)	100:1 (E:T)
Normal CD4$^+$+Normal CD8$^+$	25 ± 5	30 ± 11
Normal CD4$^+$ alone	-3 ± 1	1 ± 2
Normal CD8$^+$ alone	0 ± 2	5 ± 1
Mafo-CD4$^+$ alone	-1 ± 4	2 ± 1
Mafo-CD8$^+$ alone	4 ± 3	3 ± 7
Mafo-CD4$^+$+Mafo-CD8$^+$	19 ± 10	32 ± 6
Mafo-CD4$^+$+Normal CD8$^+$	43 ± 8 [***]	59 ± 7 [**]
Mafo-CD8$^+$+Normal CD4$^+$	10 ± 8	28 ± 3

Data expressed as mean % lysis±S.D (n=3 experiments). P values of the comparison between Mafo-treated cells and the normal CD4$^+$+normal CD8$^+$ combination are represented as: ***P<0.005 and **P<0.01 (paired Student's t-test). E:T=Effector:target raio

  Effect of pretreatment of CD4 and CD8 T-cells with Mafo on the generation of suppressor cells. Suppressor cells were not detected in the suppressor cell assay when CD4 or CD8 T-cells were pretreated with Mafo at the dose of 0.1μM (figure not shown).

  Effect of Mafo on the proliferative response in the MLR in the presence of cAMP. The increased proliferation in the MLR of lymph node cells treated with 0.1μM Mafo was examined in the presence of 1μM and 100μM cAMP. Interestingly, a low dose of Mafo (0.1μM) significantly reversed the inhibitory effect of 1μM cAMP. The results are shown in figure 4.

Figure 4. *Effect of Mafo on the proliferative in the MLR response in the presence of cAMP. Data expressed as mean ^{3}H-thymidine uptake±S.D (% of control, n=3 experiments). •P<0.05 compared with control MLR. *P<0.05 compared with diBu-cAMP treated MLR culture*

Discussion

Previous study was shown that the immunoaugmentation effects of CYP were at the lower concentrations that result in the inhibition of Ts cell activation (Asli *et al* 2004). It is shown that CYP is one of the most potent inhibitors of Ts cells (Matar *et al* 2002). Suppressor T-cell subsets may vary in their sensitivity to CYP, and immature precursor cells seem to be more sensitive than mature T-cells. The CYP-sensitive suppressor cells were identified as CD4 positive T-cells (Awwad & North 1989), because the effect of CYP pre-treatment of lymphocytes, *in vivo*, may be

directed predominantly at CD4 T-cells. The effect of Mafo treatment *in vitro* on the function of CD4 and CD8 T-lymphocytes was also investigated (Botzler *et al* 1997). It has been shown Mafo to enhance the acquisition of T-cell mediated antitumor responses in a variety of animal models. In this study, T-suppressor cell populations were particularly sensitive to the action of Mafo. The dose-response curve of Mafo *in vitro* in allogeneic MLR showed a pattern similar to that of CYP *in vivo* pretreated. At very low doses, between 0.01 and 1μM, Mafo increased proliferation and cytotoxic activity. The CD4 positive T-helper cells were shown to be more sensitive to Mafo than the cytotoxtic/effector (CD8 positive Tc/s) cells. In this study, CD4 or CD8 cells alone, with or without treatment of Mafo, did not generate good responses in terms of proliferation in allogeneic MLR and cytotoxic activity, while mixtures of both cells did lead to proliferative activity and the activation of cytotoxic cells. However, when Mafo-treated CD4 T-cells were mixed with normal CD8 T-cells as responder cells in a MLR there was a greater proliferative response and increased generation of CTL than when the responder cells were treated CD8 T-cells with normal CD4 T-cells. These results suggest that Mafo affects predominantly the CD4 T-cells.

These augmented responses, at low doses suggest that there is little or no DNA cross linkage. As significant cross linking of DNA by Mafo do not occur until higher doses of CYP and Mafo are reached (Hilgard *et al* 1985, Fritz *et al* 1997, Heim *et al* 2000), a mechanism other than DNA cross linkage can be suggested for the effect of Mafo on the function of T-helper cells. No other alkylating agent has been found to have such a powerful action on immunoregulation as CYP/Mafo. Also, the possibility that cAMP may be involved in the activation of lymphocytes has been studied over the years (Novak & Rothenberg 1990, Vig *et al* 2002). A variety of pharmacological agents have been used to change the intracellular cAMP levels during activation of T-cells. Th2 clones have a higher constitutive level of cAMP than Thl cells (Novak & Rothenberg 1990). However, the results obtained

from this study showed that Mafo reversed the inhibitory effect of cAMP on lymph node cell proliferation. This suggests that adenylate cyclase activation is associated with antigen responsiveness and this may indicate one or both CD4 and CD8-associated protein kinases leading the activation of nuclear transcription factors and expression of certain T-cell subsets. This is currently under investigations.

In conclusions, the results presented here indicate that Mafo acted in a similar fashion to CYP. Lower doses of Mafo increased proliferation in the allogeneic MLR, increased the generation of cytotoxic T-cells in MLR Mafo suppressed the activation of suppressor cells and affects predominantly the CD4 T-cells. At least part of the immunomodulatory properties of cyclophosphamide-like drugs can be explained by inhibitory effects of the drug on individual APC, CD4 (Th1 and Th2) and CD8 cell subpopulations at different doses. The immunopharmacology effects of Mafo at low doses on the activation of Tc and Ts cells are compatible with a shift from a Th2-like toward a Thl-like immune response.

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