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n-Butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids

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SUMMARY

Modulation of proliferative T-cell responses by *n*-butyrate has been suggested to result from direct interference with cell cycle progression. Considering the important role of antigen-presenting cells (APC) in T-cell activation, we were particularly interested in studying the impact of *n*-butyrate on these cells. We demonstrated that pretreatment of human peripheral blood mononuclear cells (PBMC) or monocytes with this agent resulted in a dose- and time-dependent downregulation of their capability to stimulate T-cell responses with a similar pattern of inhibition when this agent was present throughout the culture period. Pretreatment with *n*-butyrate was effective in preventing both alloresponses and T-cell proliferation to immobilized anti-CD3 monoclonal antibody (mAb) suggesting alteration of costimulatory function. Flow cytometric analysis revealed that interferon-γ (IFN-γ)-induced upregulation of B7-1 expression on monocytes was profoundly inhibited by *n*-butyrate. Furthermore, this agent significantly suppressed the expression of intercellular adhesion molecule-1 (ICAM-1) or lymphocyte function-associated antigen-3 (LFA-3). In contrast, constitutive as well as cytokine-induced expression of B7-2 was enhanced by *n*-butyrate. Additionally, in monocytes, but not in T cells, treatment with *n*-butyrate led to significant alteration of membrane integrity owing to apoptotic cell death. Our findings indicate that modulation of T-cell responses by *n*-butyrate could also result from altered APC function, possibly as a consequence of downregulating distinct adhesion and/or costimulatory receptors as well as of inducing apoptosis. A potential clinical relevance of short-chain fatty acids for reducing T-cell-mediated immune reactions via modulating APC function is speculated.

INTRODUCTION

n-Butyrate and related organic compounds are well known to have multiple effects in a number of malignant or non-malignant mammalian cell types. These effects include modulation of the expression of various genes, induction of cytodifferentiation and inhibition of cell proliferation.[1] This four-carbon fatty acid has also been shown to inhibit T-cell activation in response to mitogens or alloantigen.[2–5] Recent data demonstrated that beyond inhibiting primary T-cell responses, *n*-butyrate also induced a state of antigen-specific unresponsiveness when present during antigen contact, as shown in a human γ-globulin-specific T-cell clone.[6] We found that *n*-butyrate also specifically downregulated proliferative alloresponses in freshly isolated T cells.[7,8] As drugs that blocked cell cycle progression in other phases were ineffective in inducing T-cell anergy, selective blockade in G_{1a} phase during antigen contact was reported to be the critical mechanism underlying antigen-specific downregulation of T-cell responses.[6] Based on the observation that blockade of proliferation during antigen contact using monoclonal antibodies (mAb) to interleukin-2 (IL-2) and the IL-2 receptor resulted in clonal anergy, cell division has previously been proposed to play an important role in determining the outcome of a T-cell response.[9,10]

It is well accepted that for optimal T-cell activation at least two signals are required: stimulation via the T-cell receptor (TCR) by antigen associated with major histocompatibility complex (MHC) products and a second costimulatory signal provided by antigen-presenting cells (APC). Antigen contact with the TCR in the absence of costimulation has not only been shown to be insufficient for the induction of IL-2 secretion or proliferation, but also to induce a state of functional unresponsiveness to restimulation with the specific antigen.[11,12]

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Abbreviations: APC, antigen-presenting cell; FACScan, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HLA, human leucocyte antigen; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; IL-2, interleukin-2; LFA-3, leucocyte function-associated antigen-3; mAb, monoclonal antibody; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; TCR, T-cell receptor.

Support for this model comes from a number of reports demonstrating induction of clonal anergy by antigen presentation on APC lacking adequate accessory activity,[13–15] by mAb to CD3[16] or by interference with distinct costimulatory molecules during antigen contact.[17–19]

In view of this important role of APC in T-cell activation, we wished to investigate whether *n*-butyrate could also affect the stimulatory capacity of peripheral blood-derived APC, what thus could contribute to its described immunosuppressive properties. Using monocytes as a model for peripheral blood APC, we studied the effect of this agent on the expression of surface molecules, known to play important roles in induction of T-cell growth, as well as on cell viability.

MATERIALS AND METHODS

Reagents

The sodium salt of *n*-butyric acid as well as propidium iodide (PI) were obtained from Sigma Chemical Co. (St Louis, MO). mAb to CD14 (RMO52), CD16 (3G8), CD20 (HRC20) and CD11b (Bear 1) as well as fluorescein isothiocyanate (FITC)-conjugated mAb to intercellular adhesion molecule-1 (ICAM-1, 84H10) and leucocyte function-associated antigen-3 (LFA-3, AICD58) were purchased from Immunotech S. A. (Marseille, France). Anti-CD3 mAb OKT3 was obtained from Ortho Pharmaceutical Corp. (Raritan, NJ) and anti-B7-2 mAb (IT2.2) from Pharmingen (San Diego, CA). Monoclonal Ab to B7-1 (BB1), FITC-labelled mAb to human leucocyte antigen (HLA)-DR and phycoerythrin (PE)-labelled mAb to CD14 (LeuM3) were from Becton Dickinson (Mountain View, CA). Non-binding mouse IgG1 (VIAP) was kindly provided by Dr Otto Majdic (Institute of Immunology, Vienna, Austria). FITC and PE-labelled isotype-matched non-binding mouse Ig as well as FITC-labelled goat anti-mouse Ig $F(ab')_2$ fragments were obtained from An der Grub (Vienna, Austria). Human recombinant interferon-γ (IFN-γ) was purchased from Bender (Vienna, Austria).

Cell isolation

Peripheral blood mononuclear cells (PBMC) were prepared either from heparinized blood or from buffy coats by density gradient centrifugation over Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and resuspended in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM) and 10% fetal calf serum (FCS) that had been inactivated at 56° for 30 min. For isolation of T cells, PBMC were incubated for 45 min at 4° with a mixture of mAb to CD14 (RMO52), CD11b (Bear 1), CD20 (HRC20) and CD16 (3G8). Each antibody was used at a final concentration of 1 μg/ml. Subsequently, the cells were washed and antibody-loaded cells were depleted by negative magnetic selection using sheep anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway). Final T-cell preparations were greater than 98% $CD3^+$. For enrichment of monocytes, PBMC were depleted from T cells by sheep erythrocyte-rosetting. T-cell-depleted PBMC were cultured for 1–2 hr on plastic petri dishes and, subsequently, adherent cells were gently harvested using a cell scraper.

Cell culture

Mixed lymphocyte cultures (MLC) were incubated in round-bottom 96-well microtitre plates. T cells at 5×10^4/well were incubated for 5 days with precultured (see below) allogeneic stimulator cells at various T-cell: APC ratios at 37° in a 5% CO_2 atmosphere. To investigate the direct effect of *n*-butyrate on T-cell alloresponses, cultures were incubated in the presence (at various concentrations) or absence of this agent. Cultures were set up in triplicate. For assessment of DNA synthesis, 16 hr prior to harvesting, the cultures were pulsed with 1 μCi [^{3}H]thymidine (NEN, Dreieich, Germany) and incorporated radioactivity was assessed by liquid scintillation counting. Data are expressed as mean c.p.m. ± SD of triplicate cultures. For assay of costimulatory activity of APC, wells from flat-bottom 96-well microtitre plates were coated with 100 μl of anti-CD3 mAb OKT3 in phosphate-buffered saline (PBS) at 1 μg/ml for 2 hr at 37°. Then the wells were washed with PBS, and purified T-cells (5×10^4/well) were cultured for 3 days with or without irradiated precultured autologous stimulator cells. DNA synthesis was assessed as described above.

For pretreatment with *n*-butyrate, 1×10^6 stimulator cells (PBMC, monocytes) or responder T cells were incubated (in 75×12 mm tissue culture tubes) for the indicated period of time in medium with or without *n*-butyrate at various concentrations. After culture and γ-irradiation of stimulator cells (6000 rad), the cells were washed three times with medium and subsequently added to culture.

Flow cytometry

For direct immunofluorescence, the cells were preincubated for 15 min at 4° in PBS containing human γ-globulin (Immuno AG, Vienna, Austria), washed and then incubated for 30 min at 4° with the respective FITC and/or PE-conjugated mAb at saturating concentrations. For indirect immunofluorescence, cells were labelled with the respective primary antibodies at saturating concentrations, washed and then incubated with FITC-conjugated goat anti-mouse Ig $F(ab')_2$ fragments. For double staining, labelled cells were further incubated with mouse Ig (X63) to block residual binding sites of second antibody and stained with PE-labelled mAb. Immunofluorescence was analysed on a fluorescence-activated cell sorter (FACScan) flow cytometer (Becton Dickinson, Sunnyvale, CA).

To assess cell viability, cultured cells were pelleted and incubated for at least 15 min in PBS containing PI at 0·1 μg/ml.

Measurement of apoptosis

Apoptotic cell death was detected, based on the flow cytometric method described by Telford *et al.*,[20] by staining fixed cells with the DNA dye PI. Briefly, 1×10^6 cultured cells were washed with PBS and then fixed with 80% ethanol. After at least 30 min incubation at 4°, cells were washed and resuspended in PBS containing 50 μg/ml PI, 0·1 mM EDTA, 0·1% Triton-X-100 and 2 μg/ml RNase from bovine pancreas (Boehringer-Mannheim, Mannheim, Germany). Flow cytometric analysis was performed after 24 hr incubation in the dark at room temperature. For assessment of the DNA content, data were registered on a linear scale. Doublets and debris were excluded according to scatter characteristics.

Fragmentation of DNA was analysed by agarose electrophoresis of cell lysate DNA. Cultured cells (1×10^7) were washed and incubated for 60 min in 0·5 ml lysis buffer (50 mM Tris-HCl, pH 8·0, 10 mM EDTA, 0·5% Triton-X-100) at 50°. After centrifugation at 13 000 *g* for 10 min, the supernatants

containing fragmented DNA were harvested and incubated with RNase from bovine pancreas (2 μg/ml) for 60 min at 37° and for a further 60 min at 55° with proteinase K (50 μg/ml, Sigma). Subsequently, 0·1 ml 5 M NaCl and 0·7 ml isopropanol were added and the samples were incubated at −20° overnight. The precipitated DNA was then pelleted by centrifugation and washed with 70% ethanol. The pellet was air-dried and resuspended in Tris-EDTA buffer (50 mM Tris-HCl, pH 8·0, 10 mM EDTA). After addition of gel-loading buffer (0·25% bromophenol blue, 40% sucrose), the DNA samples were run on a 1·5% agarose gel, containing ethidium bromide (0·2 μg/ml), for 60 min at 85 V. DNA was visualized by exposing the gel to ultraviolet light and the gel was photographed.

RESULTS

Downregulation of the stimulatory capacity of human PBMC by *n*-butyrate

To assess a direct effect of *n*-butyrate on T-cell alloresponses, purified T cells were incubated with irradiated allogeneic PBMC in the presence or absence of this agent. According to previous results,[5,7] addition of *n*-butyrate at culture initiation dose-dependently inhibited T-cell proliferation to alloantigen with complete downregulation at 2 mM (Fig. 1a). To investigate if *n*-butyrate could alter the ability of peripheral blood APC to induce proliferative T-cell alloresponses, allogeneic PBMC were cultured for 48 hr in medium with or without *n*-butyrate. The cells were then irradiated, extensively washed and subsequently tested as stimulator cells. As observed for direct addition, pretreatment of stimulator cells with *n*-butyrate also resulted in a dose-dependent decrease in DNA synthesis (Fig. 1a). Complete downregulation was achieved when *n*-butyrate was used at a concentration of 2 mM. Table 1 summarizes the results obtained in a total of four different donor combinations.

The observed alteration of APC function might result from impaired presentation of alloantigen, e.g. owing to altered expression of MHC class II molecules, or from decreased costimulatory function. To investigate a possible role of altered costimulation, purified T cells were stimulated with immobilized anti-CD3 mAb OKT3 at 1 μg/ml (Fig. 1b). Under these conditions, T cells did not proliferate in the absence of APC, whereas the addition of untreated irradiated autologous PBMC enabled vigorous proliferation to anti-CD3 mAb. Pretreatment of autologous PBMC with *n*-butyrate for 48 hr resulted in a dose-dependent decrease of their accessory activity. As observed for direct addition of *n*-butyrate, complete downregulation of proliferation was observed at 2 mM (Fig. 1b, Table 1).

Modulation of APC-function by *n*-butyrate is time dependent

To assess the incubation period required for modulating APC function, freshly isolated allogeneic PBMC were treated with *n*-butyrate for 1–48 hr and then tested for their stimulatory capacity (Fig. 2). As shown in Fig. 2a, in cultures pretreated with *n*-butyrate at 2 mM, profound downregulation of allostimulation was achieved after 24–48 hr pretreatment, whereas a culture period of 12 hr or less did not or only slightly affected T-cell alloresponses. Similarly, the ability of PBMC to costimu-

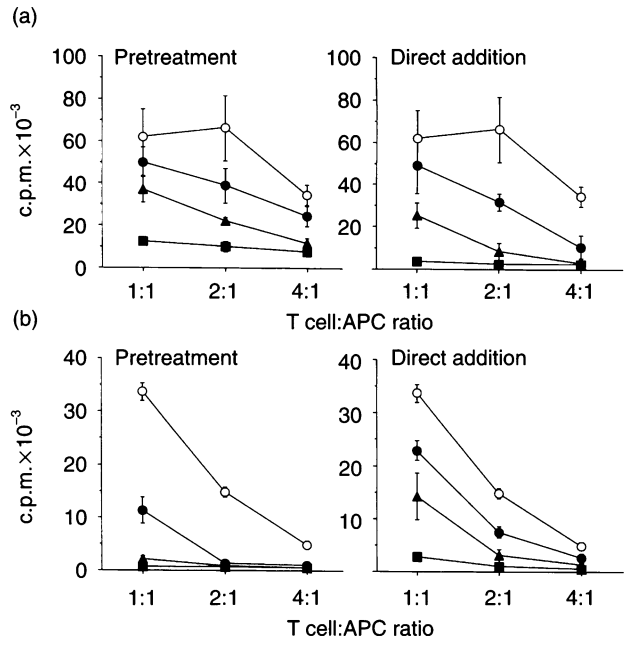

Figure 1. Effect of *n*-butyrate on the stimulatory capacity of human PBMC. Freshly isolated PBMC were cultured for 48 hr in medium alone (○) or in the presence of *n*-butyrate at 0·5 mM (●), 1 mM (▲) or 2 mM (■). Subsequently, the cells were irradiated with 6000 rad, washed and tested as stimulator cells at indicated T-cell: APC ratios. For MLC (a), purified T cells (5×10^4/well) were co-cultured for 5 days with pretreated allogeneic PBMC. For assay of costimulatory activity (b), responder T cells were stimulated for 3 days with immobilized anti-CD3 mAb OKT3 (1 μg/ml) in the presence of autologous pretreated PBMC. To assess the direct effect of *n*-butyrate on T-cell proliferation, responder T cells were stimulated with PBMC precultured in medium alone in the continuous presence of *n*-butyrate (0 mM, ○; 0·5 mM, ●; 1 mM, ▲; 2 mM, ■). Data are expressed as mean c.p.m. ± SD of triplicate cultures. C.p.m. values below 1000 were measured in cultures with no allogeneic or autologous stimulator cells either in the presence or absence of anti-CD3 mAb.

Table 1. Effect of *n*-butyrate on the stimulatory capacity of PBMC*

		% Control reactivity ± SD	
	n-Butyrate (mM)	Pretreatment	Direct addition
MLC			
	0·5	62 ± 25	82 ± 27
	1	48 ± 21	43 ± 22
	2	16 ± 6	9 ± 4
Assay for costimulation			
	0·5	51 ± 33	63 ± 22
	1	23 ± 21	33 ± 16
	2	5 ± 5	7 ± 4

*PBMC were pretreated with *n*-butyrate for 48 hr and then tested as stimulator cells at a T-cell: APC ratio of 1:1 in a MLC or in a costimulation assay. For assessment of the direct effect of *n*-butyrate on T-cell proliferation, *n*-butyrate was added at the onset of culture. DNA synthesis was assessed as described in the Materials and Methods. Mean percentage control proliferative reactivity ± SD was calculated from four (MLC) or five (costimulation assay) separate experiments.

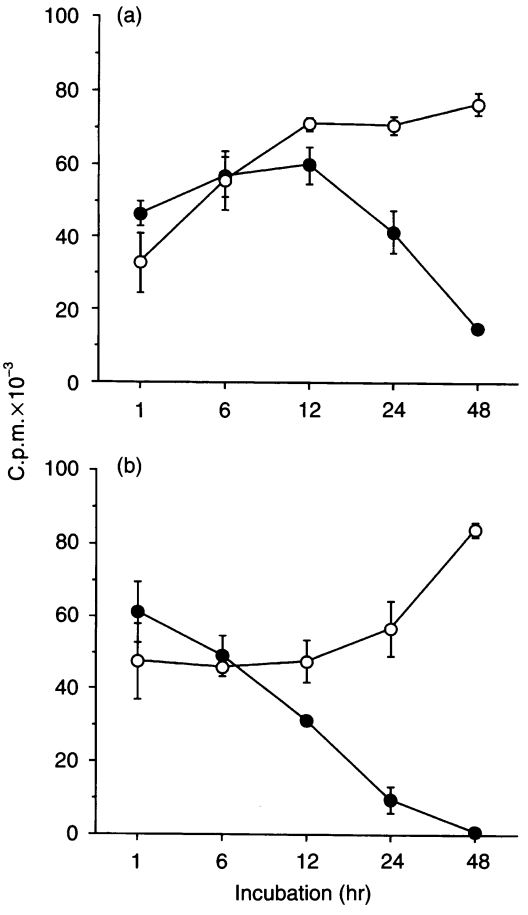

Figure 2. Kinetics of modulating APC-function by *n*-butyrate. Freshly isolated PBMC were cultured in the presence (●) or absence (○) of *n*-butyrate at 2 mM for 1–48 hr. The PMBC were then irradiated, washed and tested as stimulator cells in a MLC (a) or in a costimulation assay (b) at a T-cell: APC ratio of 1:1. Purified T cells (5×10^4) were used as responder cells. The experiment is representative of two independent experiments testing two different responders or stimulators, respectively.

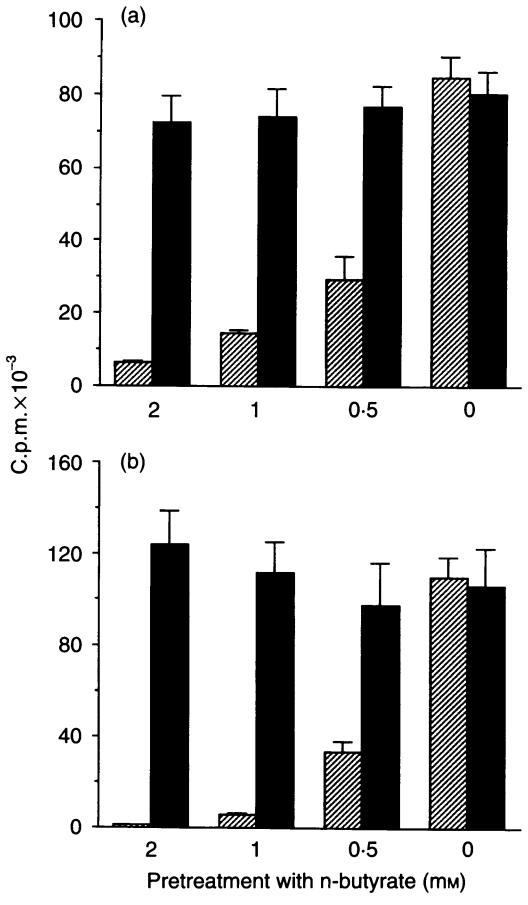

Figure 3. Downregulation of T-cell responses is not a result of suppression by *n*-butyrate-treated APC. Purified T cells (5×10^4/well) were cultured with (filled bars) or without (hatched bars) untreated PBMC at a T-cell: APC ratio of 1:1 and the effect of pretreated PBMC (5×10^4/well) from the same donor on T-cell activation was investigated. For MLC (a), allogeneic PBMC were used as stimulator cells. For analysis of costimulatory activity (b), T cells stimulated with immobilized anti-CD3 mAb (1 μg/ml) were co-cultured with autologous APC. Similar results were obtained in two other independent experiments.

late T-cell growth to anti-CD3 mAb was maximally downregulated following 24–48 hr incubation with *n*-butyrate (Fig. 2b).

Downregulation of the stimulatory ability of APC is not a result of suppression

Next we investigated whether downregulation of the stimulatory function of PBMC by *n*-butyrate is a consequence of suppression of proliferative T-cell responses by pretreated cells (Fig. 3). Purified T cells at a density of 5×10^4/well were stimulated with an equal number of unmodified allogeneic PBMC and the effect of pretreated PBMC (5×10^4/well) from the same donor on T-cell activation was investigated. Responder T cells mounted a vigorous proliferative response to untreated allogeneic PBMC. Addition of *n*-butyrate-treated stimulator cells did not significantly affect proliferative alloresponses to unmodified stimulator cells (Fig. 3a). Similar results were obtained when the number of *n*-butyrate-treated cells was increased to 1×10^5/well (data not shown). In the costimulation assay, there was also no indication for suppression of T-cell activation in response to immobilized anti-CD3 mAb (Fig. 3b).

Effect of *n*-butyrate on the stimulatory capacity of monocytes

In order to obtain further insight into the mechanisms underlying downregulation of APC function by *n*-butyrate, in the next set of experiments peripheral blood monocytes enriched from T-cell-depleted PBMC by plastic adherence were used as a model for peripheral blood APC. To examine the effect of *n*-butyrate on the capacity to promote T-cell activation, monocytes were cultured for 48 hr in medium with or without *n*-butyrate at increasing concentrations and then were added to MLC or the costimulation assay (Fig. 4). Untreated monocytes induced a vigorous alloresponse and effectively costimulated T-cell growth to immobilized anti-CD3 mAb. As described for unseparated PBMC, pretreatment of monocytes with *n*-butyrate was also found to dose-dependently decrease the stimulatory capacity in both assay systems (Fig. 4).

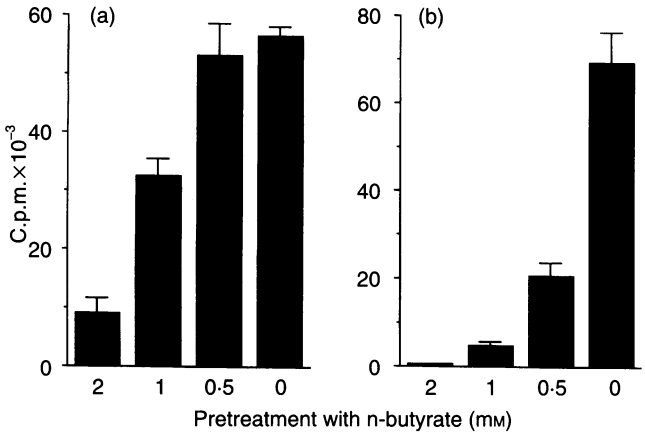

Figure 4. Effect of *n*-butyrate on the stimulatory capacity of monocytes. Monocytes isolated from T-cell-depleted PBMC by plastic adherence were cultured for 48 hr in medium with or without *n*-butyrate at the indicated concentrations. The monocytes were then irradiated (6000 rad), washed and tested at a T-cell: APC ratio of 3:1 for their allostimulatory (a) or costimulatory (b) ability. Similar results were obtained in three other independent experiments.

Modulation of surface receptor expression on monocytes by *n*-butyrate

Next, we addressed whether *n*-butyrate alters the expression of distinct surface molecules known to play important roles in T-cell activation. Marker expression was assessed on viable monocytes defined by forward scatter characteristics and positive staining with anti-CD14 mAb.

Monocytes cultured for 48 hr in medium alone or in the presence of *n*-butyrate did not or only minimally expressed B7-1 on their cell surface (Fig. 5). In agreement with previously published results,[21] incubation of these cells with IFN-γ (500 U/ml) induced significant expression of B7-1. *n*-Butyrate at 1 mM profoundly suppressed the inducible expression of this costimulatory ligand (Fig. 5a). As calculated from four independent experiments testing four different donors, the mean fluorescence intensity (MFI) in *n*-butyrate-treated cells was reduced to 52±10% (mean percentage control MFI±SD). Downregulation of cytokine-induced B7-1 was dose dependent with inhibition to values almost equivalent to non-specific antibody binding when *n*-butyrate was added at 2 mM (Fig. 5b). Furthermore, the effect of *n*-butyrate on basal as well as inducible expression of B7-2 was investigated (Fig. 5). As evaluated from four independent experiments, treatment of monocytes with *n*-butyrate at 1 mM increased the MFI by 20±25% (constitutive expression) or 72±26% (IFN-γ-induced expression), respectively. Figure 5a illustrates the results obtained in one representative experiment. Evaluation of dose dependency revealed that maximal induction of B7-2 expression was achieved when *n*-butyrate was added at 1 or 2 mM, respectively (Fig. 5b).

We then investigated the effect of *n*-butyrate on the expression of ICAM-1 and LFA-3 (Fig. 6). Constitutive as well as IFN-γ-induced expression of these surface receptors was found to be significantly inhibited by *n*-butyrate at 1 mM (Fig. 6a). As calculated from four independent experiments, basal or cytokine-induced expression of ICAM-1 was decreased to 81±11% or 73±11% and expression of LFA-3

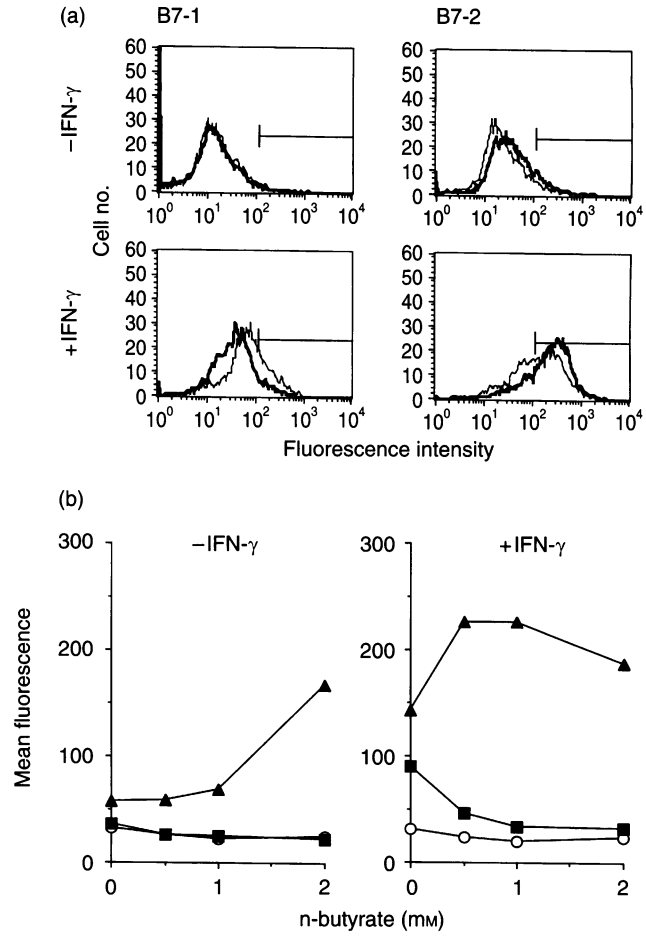

Figure 5. Effect of *n*-butyrate on the expression of B7-1 and B7-2. Monocytes were cultured for 48 hr in the presence (bold line) or absence (thin line) of *n*-butyrate at 1 mM in medium with or without IFN-γ at 500 U/ml (a). Subsequently, the cells were harvested and stained with the respective primary mAb followed by FITC-conjugated goat anti-mouse Ig $F(ab')_2$ fragments. Viable monocytes were gated according to scatter characteristics and positive staining with PE-labelled anti-CD14 mAb. The markers indicate the position of control staining with isotype matched control antibody. Similar results were obtained in cells from three other donors. For evaluation of dose dependency (b), *n*-butyrate was used at various concentrations in the presence or absence of IFN-γ (500 U/ml) and then monocytes were harvested and stained with control antibody (○) anti-B7-1 mAb (■) or anti-B7-2 mAb (▲). Similar results were obtained in a separate experiment.

to 73±11% or 71±8% of the MFI measured in control cultures (incubated in the absence of *n*-butyrate). Whereas maximal inhibition of ICAM-1 expression was observed at 0·5 mM with decreased efficacy at 1 or 2 mM, modulation of LFA-3 expression was clearly dose dependent with maximal suppression at 1 or 2 mM (Fig. 6b).

Finally, the effect of *n*-butyrate on the expression of HLA-DR on viable monocytes was assessed (data not shown). The influence of *n*-butyrate on basal or IFN-γ-induced HLA-DR expression was donor dependent. In four out of eight donors, HLA-DR expression was significantly increased, whereas in the other four donors, this fatty acid impaired expression of MHC class II molecules (data not shown). In

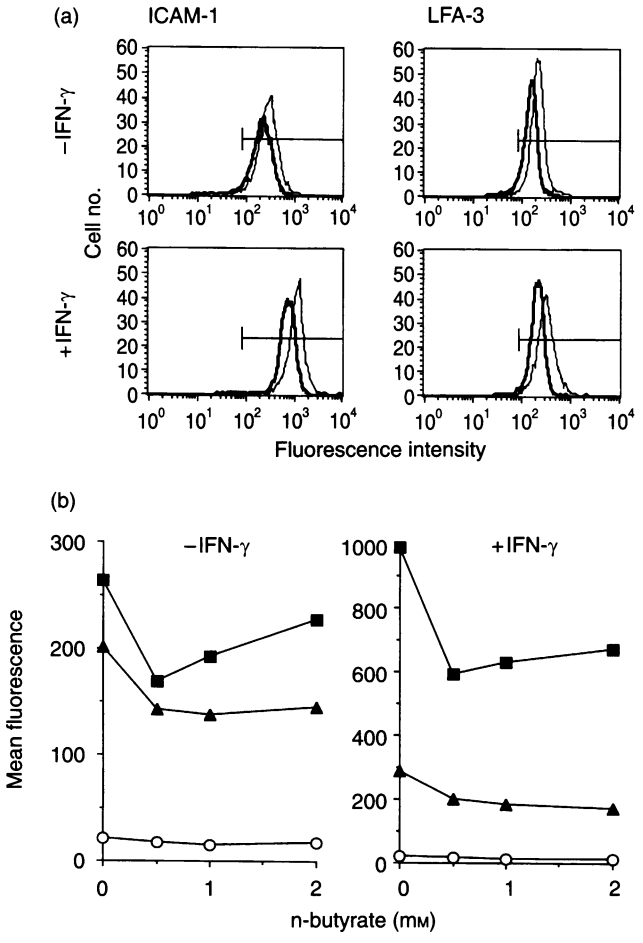

Figure 6. Effect of *n*-butyrate on the expression of ICAM-1 and LFA-3 on monocytes. Monocytes were cultured in medium with (bold line) or without (thin line) *n*-butyrate (1 mM) and/or IFN-γ (500 U/ml) and then stained with the respective labelled antibodies (a). Surface marker expression on viable monocytes gated according to scatter characteristics and positive staining with anti-CD14 mAb is depicted. The markers indicate the position of control staining with FITC-labelled control antibody. Similar results were obtained in three other donors. For assessment of dose dependency (b), cells were incubated with *n*-butyrate at increasing concentrations in the presence or absence of IFN-γ and then stained with control antibody (○), anti-ICAM-1 mAb (■) or anti-LFA-3 mAb (▲). The data from one of three representative experiments are depicted.

contrast to the differential behaviour of HLA-DR expression, the allostimulatory capacity of monocytes was clearly downregulated in all donors.

Induction of apoptosis in monocytes by *n*-butyrate

To examine the possibility that altered costimulatory activity is also related to decreased cell viability in *n*-butyrate-treated cultures, cultured monocytes were stained with PI (Fig. 7). After 48 hr culture in medium alone, a small percentage of non-viable monocytes was found as indicated by positive staining with PI and by a substantial decrease of the cell size. As shown in Fig. 7a, addition of *n*-butyrate at 2 mM clearly augmented the percentage of dead cells. As calculated from eight independent experiments testing eight different donors, induction of cell death by *n*-butyrate was dose dependent with an increase in the percentage of PI^+ cells from $17 \pm 5\%$ (mean percentage ± SD) in control cultures to $23 \pm 5\%$, $34 \pm 9\%$ and $46 \pm 15\%$ following 48 hr culture with *n*-butyrate at 0·5, 1 or 2 mM, respectively.

To investigate if the observed alteration of membrane integrity was a consequence of apoptotic cell death, monocytes, which had been cultured for 48 hr in the presence or absence of *n*-butyrate at 2 mM, were stained with PI following fixation with ethanol. This method has previously been shown to discriminate apoptotic cells as their DNA has a reduced ability for stain uptake.[20] As shown in Fig. 7b, in control culture, a small percentage of the cells was apoptotic as indicated by their subdiploid DNA content (A_0 region). Treatment with *n*-butyrate at 2 mM led to an approximate threefold accumulation of events in the A_0 region and a concomitant decrease in the percentage of cells in the G_0/G_1 compartment (Fig. 7b).

Induction of apoptotic cell death by *n*-butyrate was further confirmed by agarose electrophoresis of low-molecular-weight DNA isolated from precultured monocytes. As shown in Fig. 7c, monocytes incubated for 48 hr in medium alone showed low but significant levels of non-random DNA laddering, characteristic of apoptotic cell death. The amount of low-molecular-weight DNA and the extent of DNA fragmentation were clearly increased following pretreatment with *n*-butyrate at 2 mM (Fig. 7c).

In order to illustrate the contribution of *n*-butyrate-induced cell death to downregulation of the stimulatory capacity of monocytes, the calculated absolute number of added viable stimulator cells was plotted against the proliferative responses in MLC (Fig. 8a) or in the costimulation assay (Fig. 8b). As discussed above, culture of monocytes in the presence of *n*-butyrate led to a dose-dependent reduction of the number of viable stimulator cells with an approximate two- to threefold reduction when *n*-butyrate was added at 2 mM. When proliferative responses induced by pretreated APC were compared with those of untreated APC on the basis of viable stimulator cells present in either culture, the stimulatory capacity of *n*-butyrate-treated APC was significantly lower than that of an equal number of untreated cells. Hence, downregulation of T-cell activation in this model cannot be attributed to cell death alone.

Pretreatment of responder cells with *n*-butyrate

To investigate if pretreatment of T cells with *n*-butyrate also affects proliferative responses, T cells were incubated in medium with or without *n*-butyrate for 48 hr, washed and then stimulated with allogeneic PBMC or with immobilized anti-CD3 mAb plus autologous PBMC. Pretreatment of responder T cells with increasing concentrations of this agent did not significantly affect proliferative T-cell responses (data not shown). Furthermore, in T cells, cell viability was not or was only slightly affected by 48 hr treatment with *n*-butyrate. As assessed in four different donors, the percentage of PI^+ cells ± SD made up $6 \pm 3\%$, $7 \pm 3\%$, $9 \pm 4\%$ or $11 \pm 5\%$ following treatment with *n*-butyrate at 0, 0·5, 1, or 2 mM, respectively.

DISCUSSION

The purpose of the present study was to investigate whether modulation of T-cell responses by short-chain fatty acids could

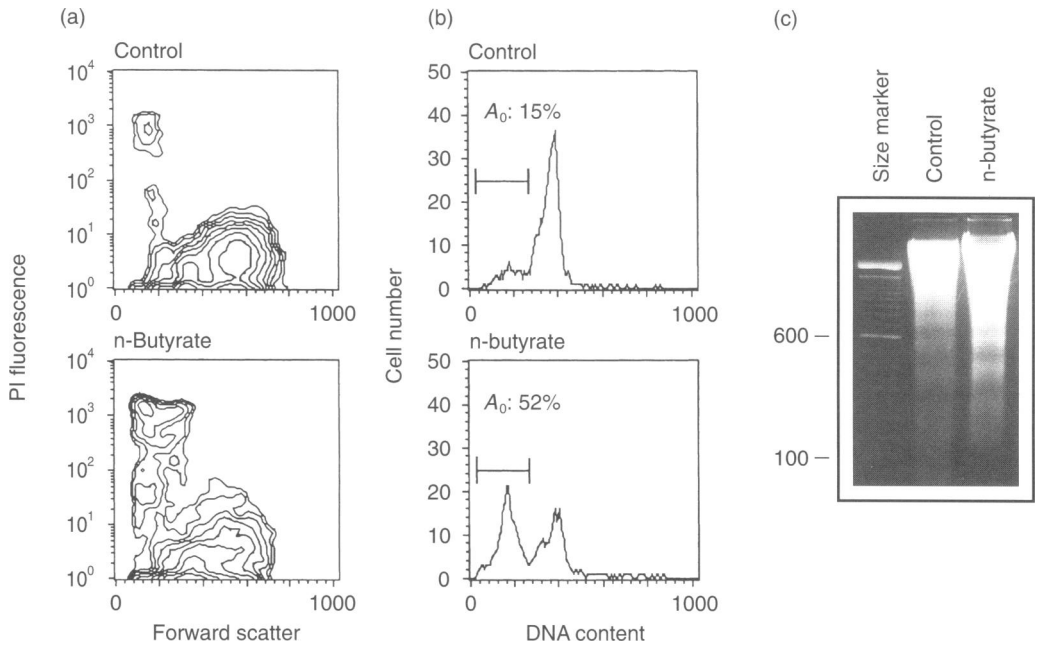

Figure 7. Influence of *n*-butyrate on cell viability. Freshly isolated monocytes were cultured for 48 hr in medium with[1] or without *n*-butyrate at 2 mM.[2] For measurement of cell viability (a), the cells were washed and resuspended in PBS containing PI at 0·1 μg/ml. After 15 min incubation at room temperature, flow cytometric analysis was performed. Data are plotted as forward light scatter versus PI uptake. For detection of apoptosis (b), cultured monocytes were stained with PI following fixation with ethanol. DNA fluorescence histograms are depicted. Doublets and debris were excluded according to forward and side scatter characteristics. The percentage of apoptotic cells is indicated (A_0). The shown experiment is a representative of a total of five independent experiments. For assessment of DNA fragmentation (c), 1×10^7 monocytes cultured for 48 hr in the presence or absence of *n*-butyrate (2 mM) were lysed, and low-molecular-weight DNA was run on an agarose gel as described in the Materials and Methods. As size marker, a 100-bp molecular weight marker (100–2072 bp) was used. Similar results were obtained in three other separate experiments.

be related to alteration of the stimulatory function of APC. We found that pretreatment of PBMC or of peripheral blood monocytes with *n*-butyrate led to a dose- and time-dependent loss of their capability to induce T-cell alloresponses with a similar pattern of inhibition when this agent was added directly to culture. As the ability to costimulate T-cell growth to suboptimal concentrations of immobilized anti-CD3 mAb was also impaired by *n*-butyrate, we suggest that modulation of T-cell responses appears, at least partially, to be caused by the inability of pretreated cells to provide essential costimulatory activity. The observation that pretreated APC did not affect T-cell activation stimulated with unmodified APC argues against active downregulation of T-cell responses. Pretreatment of responder T cells with *n*-butyrate for 48 hr did not affect their ability to be activated in subsequent MLC or assay for costimulation, suggesting that, in contrast to APC, this agent might not change the functional state of T cells. The possibility of reversible alteration of T-cell function requiring the continuous presence of *n*-butyrate, however, cannot be excluded. The finding that preincubation of T cells with *n*-butyrate did not influence proliferative responses in subsequent T-cell activation assays further argues against the possibility of direct carryover of this agent into subsequent culture. This is also supported by the observation that an incubation, shorter than 12 hr, of APC with *n*-butyrate did not result in reduced T-cell reactivity.

Investigating the impact of *n*-butyrate on the expression of various surface molecules known to play important roles in the process of T-cell activation by mediating intercellular contact and/or by delivering essential costimulatory activity, we found that *n*-butyrate profoundly inhibited inducible expression of B7-1 on viable monocytes and partially suppressed constitutive as well as inducible expression of ICAM-1 and of LFA-3 on these cells. B7-1, ICAM-1 and LFA-3 have been shown to provide critical costimulation upon interaction with their counter-receptors on T cells CD28, LFA-1 or CD2, respectively.[22–25] There is further evidence that distinct costimulatory receptors might synergistically costimulate T-cell activation, such as B7-1 and ICAM-1[26,27] or B7-1 and LFA-3.[28,29] Synergistic action of distinct costimulatory molecules is also supported by the observation that complete inhibition of proliferation to alloantigen can be achieved only by combined blockade of two or more costimulatory receptors.[30] Accordingly, it is tempting to speculate that the simultaneous downregulation of B7-1, ICAM-1 and LFA-3 by *n*-butyrate might be an effective mechanism underlying the decreased ability of pretreated APC to stimulate T-cell responses.

In contrast to B7-1, constitutive as well as IFN-γ-induced expression of B7-2 on monocytes was significantly increased by *n*-butyrate. Regarding the profound downregulation of the stimulatory function of APC, this might suggest that in our model expression of B7-2 alone is insufficient to provide effective costimulation. The relative contribution of B7-1 and B7-2 to costimulation of T-cell growth is still unclear and might critically depend on the experimental model tested. In

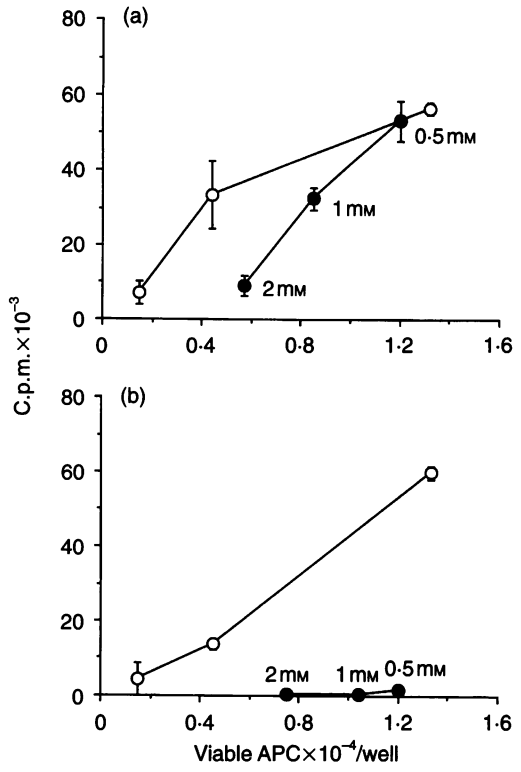

Figure 8. Contribution of *n*-butyrate-induced alteration of cell viability in monocytes to downregulation of APC function. Purified T cells (5×10^4/well) were co-cultured with monocytes that had been precultured for 48 hr in culture medium alone (○) or in the presence of *n*-butyrate at indicated concentrations (●). Untreated stimulator cells were added at a T-cell: APC ratio of 3:1, 9:1 and 27:1, and pretreated cells at a T-cell: APC ratio of 3:1. The actual number of added viable stimulator cells was calculated according to negative PI staining and scatter characteristics. The stimulatory capacity of precultured monocytes was tested in a MLC (a) or in a costimulation assay (b), respectively. Data are reported as mean c.p.m. ± SD. Similar results were obtained in another two (MLC) or three (costimulation assay) separate experiments.

some instances, B7-1 costimulation has been shown to be superior to B7-2 costimulation,[31,32] even though many other studies argue for a predominant role of B7-2 versus B7-1, which might be explained by a differential pattern of expression, i.e. constitutive expression of B7-2 on some APC and rapid induction of this marker on others.[33–36] Considering the decreased expression of the two adhesion receptors ICAM-1 and LFA-3, the inability to costimulate T-cell growth despite considerable levels of B7-2 expression could also result from impaired interaction of this costimulatory molecule with its counter receptor owing to altered intercellular contact.

There is recent evidence that in monocytes, expression of B7 might be insufficient to confer potent costimulatory function.[37] In these cells an important role of a novel, yet unidentified, costimulatory molecule has also been suggested, even though the possibility of costimulation via ICAM-1 could not be excluded.[37] In line with these results, blocking mAb to B7-1 and/or B7-2 did not or only slightly inhibited proliferation in the costimulation assay (our unpublished results). Therefore, an involvement of additional costimulatory molecules might well play a role in downregulating APC function of monocytes by *n*-butyrate.

We further described that pretreatment of monocytes with *n*-butyrate results in alteration of membrane integrity, which correlated well with the occurrence of apoptotic cell death. Our observation that distinct levels of apoptosis could also be detected in control cultures was in line with the previous finding that long-term culture of peripheral blood monocytes in culture medium supplemented with FCS resulted in significant T-cell death and DNA fragmentation.[38] Induction of cell death in monocytes might substantially contribute to altered APC function of peripheral blood monocytes, but was demonstrated to only partially contribute to the observed degree of reduction of the stimulatory function.

n-Butyrate has earlier been demonstrated to induce apoptotic cell death in distinct T-cell types, e.g. in colorectal tumour cell lines[39,40] or in distinct human myeloid leukaemic cell lines.[41,42] In HL-60 and U937 cells, *n*-butyrate-induced apoptosis has been shown to correlate with differentiation along the monocytic pathway.[42] The susceptibility to *n*-butyrate-induced apoptosis, however, critically depends on the cell type tested. Thus, some cell lines such as the myeloblastic KG1 and the NB4 promyelocytic leukaemic cell lines have been described to be resistant to apoptosis induced by a monosaccharide ester of *n*-butyric acid.[42] Furthermore, *n*-butyrate has recently been reported to delay apoptosis in neutrophils.[43] Differences in the capability of *n*-butyrate to induce apoptosis is further supported by our observation that, in comparison to monocytes, T cells are clearly more resistant to induction of cell death.

The herein described downregulation of the stimulatory capacity of peripheral blood APC is in contrast to previous reports investigating the effect of this agent on distinct immortalized cell lines. Thus, *n*-butyrate has been reported to increase the allostimulatory ability of various Epstein–Barr virus (EBV)-positive Burkitt lymphoma lines, which was suggested to result from increased surface expression of MHC class II molecules.[44] In our assay system, however, allogenicity of *n*-butyrate-treated monocytes appeared not to correlate with an effect of this agent on the expression of MHC class II molecules. Whereas the allostimulatory capacity was similarly downregulated in all donors, basal as well as cytokine-induced expression of HLA-DR was up- or downregulated apparently as a result of donor dependency. Thus, impaired APC function of monocytes was even observed when expression of HLA-DR molecules was substantially increased. Furthermore, pretreatment of a rat colon carcinoma cell line with *n*-butyrate has recently been shown to result in enhanced immunogenicity and thus good protection against a challenge with untreated tumour cells.[45] Induction of ICAM-1 expression and increased expression of MHC class I molecules in *n*-butyrate-treated cancer cells was thought to contribute to this effect.[45] The impact of *n*-butyrate on APC-function might therefore critically depend on the cell type tested.

There is recent evidence that, in contrast to peripheral blood monocytes, monocytes isolated from intestinal lamina propria cannot effectively support T-cell proliferation, which was considered to contribute to intestinal T-cell hyporesponsiveness.[46] Interestingly, altered APC-function was found to be associated with impaired surface expression of the accessory receptors ICAM-1, LFA-3 and B7-1. Regarding the high levels

of *n*-butyrate in the colon (millimolar levels), where it is produced as a major product of microbial fermentation of carbohydrates,[47] and in view of our observation that this agent is able to affect the stimulatory function of peripheral blood APC, possibly also because of altered expression of distinct adhesion and/or costimulatory receptors, it is tempting to speculate a potential physiological role of short-chain fatty acids in regulating the function of intestinal APC, which could therefore contribute to the formation of the particular properties of the immune system in the gut.

n-Butyrate or related compounds have previously been suggested to have potential clinical relevance for suppressing allograft rejection *in vivo*. Supplementation of drinking water with *n*-butyric acid has been shown to result in modest but significant prolongation of skin allograft survival in mice.[5] Low efficacy of *n*-butyrate *in vivo*, however, might be explained by its very short metabolic half-life and thus insufficient levels in blood. One promising approach to achieve pharmacologically active concentrations could be the use of longer acting and/or more effective derivatives of *n*-butyrate.[48] Our finding that *n*-butyrate profoundly affects costimulation might warrant further studies concerning the potential clinical application of this agent for reducing T-cell-mediated immune reactions by means of modulating APC function.

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