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A sigma ligand, SR 31747A, potently modulates Staphylococcal enterotoxin B-induced cytokine production in mice

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SUMMARY

Sigma receptors originally described in distinct regions of the central nervous system are expressed on cells of the immune system. A sigma ligand, SR 31747A, was observed here to inhibit *in vitro* the Staphylococcal enterotoxin B (SEB)-driven lymphocyte proliferation. In mice, the drug confers a potent protection against the lethality induced by SEB, stimulates the SEB-induced serum release of interleukin (IL)-10 and inhibits at the same time the systemic release of IL-2, IL-4, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-6 and tumour necrosis factor-α (TNF-α). The enhancement of IL-10 production by this compound is also effective in nude mice treated with SEB, indicating that IL-10 of T-cell origin is not involved in this process. The finding that a sigma ligand protects against the SEB-induced toxicity provides insights into the clinical use of this family of compounds, particularly in food poisoning and septic shock where Staphylococcal enterotoxins are involved. The observation that this compound stimulates IL-10 synthesis indicates that it could be a potent regulatory agent of chronic inflammatory diseases.

INTRODUCTION

SR 31747A (*N*-cylohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride), is a new immunomodulator eliciting high affinity for sigma receptors expressed on lymphocytes.[1] These receptors originally described in guinea-pig brain,[2] were further identified in peripheral organs, including endocrine-related structures,[3] gastrointestinal tract,[4] liver[5] and kidney.[6] Recently, they have been found to be distributed on mouse lymphocytes and rat splenocytes[1,7] and also on human peripheral blood mononuclear cells.[8] Moreover, Liu *et al.*,[9] using a panel of sigma agonists, observed a high correlation between drug binding potency at sigma sites and the ability of these drugs to modulate concanavalin A-induced splenocytes proliferation. This result strongly indicates that sigma receptors expressed on splenocytes are functional and that sigma agonists may have direct effects on immune cells, suggesting that sigma receptors may be considered as targets for immunomodulation. The sigma ligand SR 31747A (recently described) presents strong immunosuppressive effects on mitogen-induced mouse and human T-cell proliferation in nanomolar concentrations, with a potency dramatically higher than that of the sigma agonists already known. We showed that this ligand exhibits unique pharmacological profile. In mice, it has been observed to inhibit several cellular immune responses such as graft-versus-host disease and delayed-type hypersensitivity.[10] Furthermore, it markedly suppresses the lipopolysaccharide (LPS)-induced production of interleukin (IL)-1, IL-6, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α),[11,12] stimulating at the same time the production of IL-10.[12]

In this study, we observed that SR 31747A, which inhibits *in vitro* the staphylococcal enterotoxin B (SEB)-driven lymphocyte proliferation in the nanomolar range, was a strong protector against the lethality induced in mice by SEB and D-galactosamine (D-Gal). Considering the role played by some cytokines in this model, we evaluated the impact of this compound on these cytokines, particularly IL-10 regarded as a life-preserving cytokine.[13,14] These data support the concept that SR 31747A, displaying high affinity for sigma receptors expressed on lymphocytes, potently modulates immune functions and has to be considered as a potential therapeutic agent.

MATERIALS AND METHODS

Reagents

D-Gal, dexamethasone 21-phosphate and SEB were purchased from Sigma Chemical Co. (St. Louis, MO). The endotoxin level of SEB was 0·00029% as determined by the *Limulus* amoebocyte lysate assay (Bioproducts, Walkersville, MD). Cyclosporin A was kindly provided by Sandoz (Rueil Malmaison, France). SR 31747A was synthesized at the Chemistry Department of Sanofi Recherche (Montpellier, France). These reagents were dissolved in phosphate-buffered saline (PBS), except for SR 31747A that was dissolved in 5% ethanol, 5% tween 80 and 90% H_2O, and were injected either intravenously (i.v.) or intraperitoneally (i.p.) at the indicated doses.

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Culture medium

Complete cell culture medium was RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories), 2 mM L-glutamine, 5×10^{-5} M 2-βmercaptoethanol (Merck, NoGent, Sur Marne, France), 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratories), 15 mM PIPES (Sigma Chemical Co.) and the pH was adjusted to 6·6.

Isolation of cells

Cell suspensions were prepared from spleens using a 80-gauge stainless steel mesh. Red cells were first lysed by a short hypotonic shock and the remaining cells were washed twice in culture medium before use. Cell viability determined by trypan blue exclusion always exceeded 95%.

In vitro *T-cell proliferation*

Splenocytes were cultured in 96-well flat-bottomed plates (Falcon Becton Dickinson, Lincoln Park, NJ) in quadruplicate at 4×10^5 cells/well with 1 μg/ml of SEB. Cells were cultured in complete medium for 4 days, then pulsed with 1 μCi/well [^{3}H]thymidine ([^{3}H]TdR) (Amersham, les Ulis, France), and harvested 4 hr later on glass fibre papers using a skatron harvester system (Pharmacia-LKB, Piscataway, NJ). Incorporated radioactivity was measured by using a betaplate liquid scintillation spectrometer (Pharmacia-LKB). Results are expressed as mean counts per minute ± SD.

Mice

Six to 8-week-old normal or nude BALB/c female mice were purchased from IFFA-CREDO (L'arbresle, Lyon, France). All animals rested for 7 days prior to the onset of treatments.

Cytokine determinations

Mice were injected with either SR 31747A, dexamethasone or their vehicles only, by i.p. route, 30 min before D-Gal (20 mg/mouse, i.p.) and SEB (10 μg/mouse, i.v.). Blood samples were obtained by retro-orbital puncture at different times after SEB injection. Subsequently, samples were centrifuged, and sera were collected and stored at −80° until use for cytokine determination. IL-2 and IL-10 were determined using enzyme-linked immunosorbent assay (ELISA) kits from Genzyme (Genzyme Corp, Cambridge, MA). IL-4, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), TNF-α and IFN-γ levels were determined using ELISA kits from Endogen (Endogen Inc, Boston, MA). The assays were performed as indicated by the manufacturer's instructions. The data are expressed as pg/ml following calibration with a reference standard. The detection limit was 15 pg/ml for every cytokine, except for GM-CSF, whose sensitivity was 5 pg/ml.

Lethal endotoxinic shock

Animals were treated with either SR 31747A, dexamethasone or cyclosporin A, administered by i.p. route. Thirty min later, they received SEB and D-Gal as described previously. In these experiments, 10 animals were assigned to each drug dose and lethality was scored 48 hr later.

Statistical analysis

Statistical significance was determined by the RS/1 multicompare procedure using the one-way analysis of variance and a Dunnett's test for multiple comparisons with a common control group.

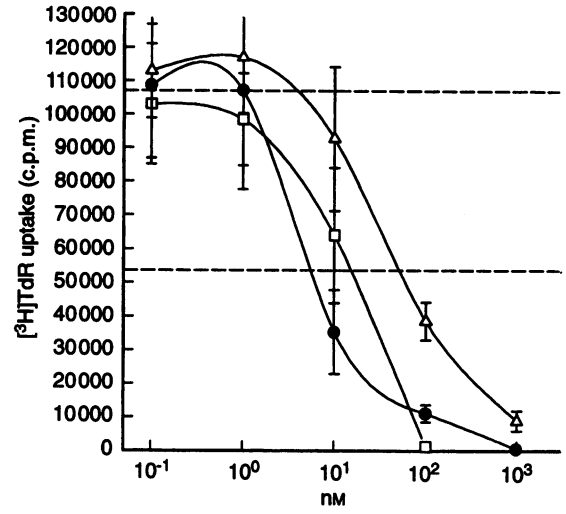

Figure 1. SR 31747A (●), cyclosporin A (□), dexamethasone (△) inhibit the proliferation of splenocytes induced by SEB in BALB/c mice. Spleen cells were isolated from naive BALB/c mice and cultured (4×10^5/well) for 96 hr in the presence of 1 μg/ml SEB. [^{3}H]TdR was incorporated during the last 4 hr. Vertical bars show standard deviation from quadruplicate cultures.

RESULTS

Effect of SR 31747A on SEB-driven lymphocyte proliferation

In splenocytes isolated from naive mice, 1 μg/ml SEB induced a strong proliferation, detectable by [^{3}H]TdR incorporation on day 4 of the culture. The sigma ligand SR 31747A, cyclosporin A and dexamethasone added at the time of SEB stimulation produced dose-dependent inhibitions of this proliferative response (Fig. 1). [^{3}H]TdR incorporation was decreased to 50% of control values in the presence of 6 nM SR 31747A, 15 nM cyclosporin A or 50nM dexamethasone. A reduction in the viability of splenocytes does not appear to be a likely cause for suppression of proliferation by SR 31747A. Indeed, there was no difference in the viability of cells cultured for 4 days in this assay in the presence or absence of 10^{-7} M of the drug, although the mitogenic response was suppressed to near background levels at this dose (48·6% ± 0·2% viable cells in the control group versus 49·5% ± 1·21% viable cells in the SR 31747A group, $n = 5$, as determined by flow cytometry).

SR 31747A prevents the lethal shock induced by SEB in D-galactosamine-sensitized mice

All (100%) mice receiving 10 μg SEB and 20 mg D-Gal, died within 16–24 hr. Treatment with SR 31747A by i.p. route, 30 min before SEB challenge, caused a strong and dose-dependent inhibition of lethality: 70% and 90% of mice survived when treated at 25 mg/kg or 100 mg/kg dose levels, respectively (Fig. 2a). Similarly, administration of cyclosporin A at 25 mg/kg resulted in a 100% survival, whereas dexamethasone at 2 mg/kg protected 100% of the mice. Life-preserving effect was also observed when SR 31747A was

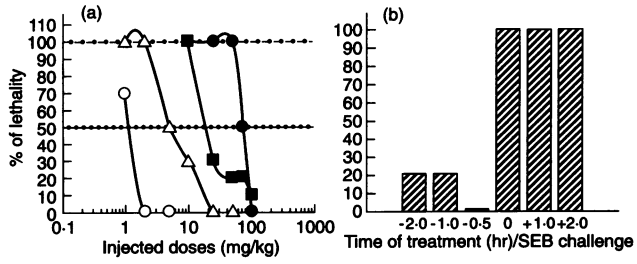

Figure 2. SR 31747A, cyclosporin A and dexamethasone protect mice against the lethal effect of SEB in D-Gal-sensitized mice. (a) SR 31747A (■, i.p. or ●, p.o.), cyclosporin A (Δ, i.p.) or dexamethasone (○, i.p.) were injected in groups of 10 BALB/c mice 30 min before D-Gal (20 mg/mouse, i.p.) and SEB (10 μg/mouse, i.v.). Lethality was scored 48 hr later, since no mortality was observed thereafter. (b) SR 31747A (50 mg/kg, i.p.) was injected i.p. at different times before, simultaneously with, or after SEB and D-Gal challenge. Lethality was scored 48 hr later.

administered at a 100 mg/kg dose by p.o. route, 1 hr before SEB and D-Gal. The protective effect was observed when SR 31747A was administered before SEB challenge. Given simultaneously with or after SEB challenge, the ability of SR 31747A to protect disappeared (Fig. 2b).

SR 31747A differentially regulates SEB-induced cytokines in BALB/c mice

Injected i.v. in mice, SEB induces the systemic release of a wide range of cytokines. In this experiment, we questioned whether SR31747A-induced protective effects might be mediated through inhibition of the production of some cytokines potentially involved in the pathology.

Shortly after SEB administration, we observed that IL-2, IL-4, IL-10 and GM-CSF appeared in the serum (Fig. 3), peak levels being found 1·5 hr post-SEB administration. Surprisingly, IL-6, IFN-γ and TNF-α appeared in two phases: these cytokines were first detected at low levels between 1·5 hr and 3 hr post-SEB administration, then disappeared from the serum, and were detected again at very high levels 12 hr after SEB injection, when mice showed the first signs of illness. This late increase in cytokine levels appeared to be very reproducible. This effect observed in mice receiving both SEB and D-Gal together could actively contribute to the lethal syndrome.

Injected i.p. at a 50 mg/kg dose, SR 31747A significantly reduced the release of most of these cytokines, particularly IL-4, GM-CSF, IL-6 and TNF-α (Fig. 3). Concerning IL-10, SR 31747A treatment resulted 1·5 hr after SEB injection in a rapid and transient increase of IL-10 levels (300 ± 40 pg/ml in SR 31747A-injected mice versus 162 ± 32 pg/ml in mice pretreated with vehicle alone, $P < 0{\cdot}01$). Then, IL-10 levels returned to near background values at 3 hr.

T cells were not involved in the enhancement of IL-10 release under SR 31747A treatment

IL-10 is secreted by different cell subsets including $CD4^+$ cells, B cells and macrophages. We thus investigated which cells might account for IL-10 production. For this reason, the effects of SR 31747A were evaluated in D-Gal-sensitized nude mice treated with SEB (Fig. 4). 1·5 hr after SEB injection, low levels

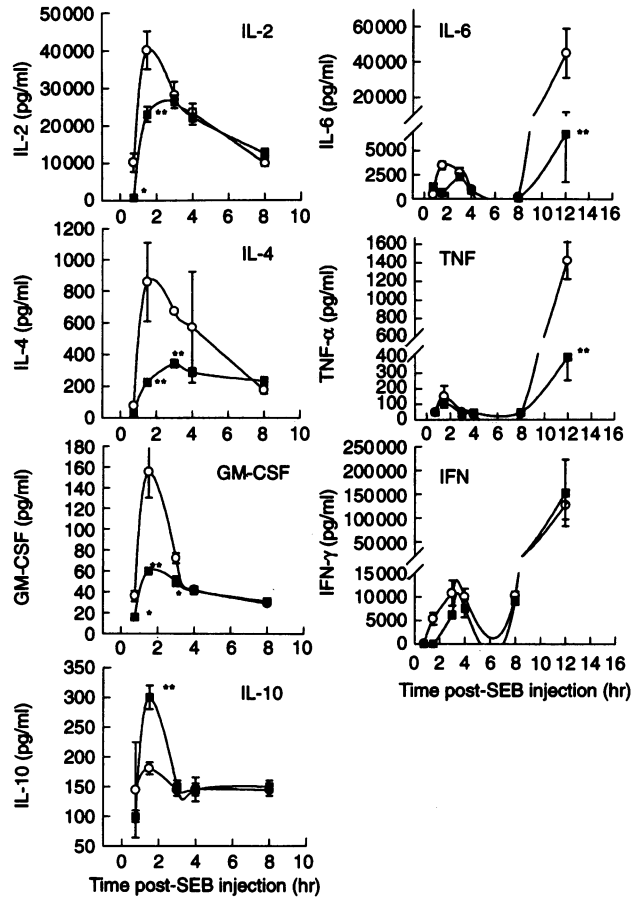

Figure 3. Effect of SR 31747A preteatment on the kinetics of SEB-induced IL-2, IL-4, IL-6, IL-10, IFN-γ, GM-CSF and TNF-α production in BALB/c mice. Groups of six to 10 mice were treated with SR 31747A (50 mg/kg, i.p.) (■) or vehicle only (○), then with SEB (10 μg/mouse, i.v.) and D-Gal (20 mg/mouse, i.p.) 30 min later. Cytokine serum levels were measured at the indicated time points after SEB challenge. Data are means ± SEM. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. Results are representative for one out of three experiments involving five animals/group.

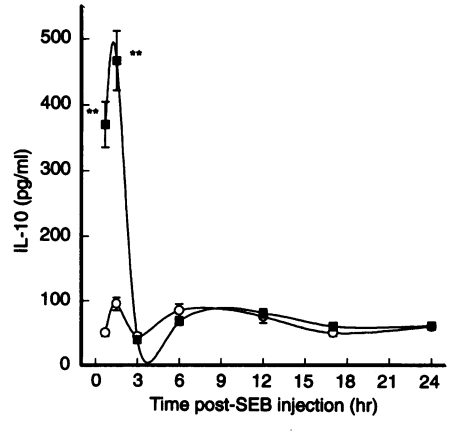

Figure 4. T cells are not required for SR 31747A to increase IL-10 release. IL-10 levels were measured in D-Gal-sensitized nude mice treated with SEB. Five mice were treated in each group with SR 31747A (■) or vehicle only (○), as indicated in Fig. 3. Data are means ± SEM. $**P < 0{\cdot}01$.

of IL-10 were detected in the circulation of these T-deficient mice (95 pg/ml ± 5 pg/ml). A pretreatment with SR 31747A induced a strong IL-10 peak (460 pg/ml ± 50 pg/ml), coinciding with that observed in normal mice. In contrast, IL-6, IFN-γ and TNF-α were not detected at significant levels at any time points in the serum of these mice (data not shown).

DISCUSSION

Previous results have shown that a sigma ligand SR 31747A,[1] displayed immunosuppressive properties both *in vitro* by inhibiting mitogen-induced mouse or human lymphocyte proliferation,[10] and *in vivo* by including local and systemic graft-versus-host diseases and delayed-type hypersensitivity, on various cellular immune responses.[10] In this study, we observed that this compound was also a strong inhibitor of the lymphocyte proliferation induced in mouse splenocytes by SEB. This effect elicited in the nanomolar range was consistent with a receptor-mediated process, as high-affinity binding sites of [^{3}H]TdR SR 31747A have been identified on spleen cells ($K_d = 0{\cdot}66$ nM).[1] T-cell activation by Staphylococcal enterotoxins requires T-cell receptor (TCR) and CD28-costimulatory signals delivered by the antigen-presenting cell (APC).[15] Hence the inhibition of T-cell proliferation by SR 31747A may result either from a direct effect on T cells or from an indirect effect on the APC. Although we do not have the response yet, we favour the hypothesis that SR 31747A might directly affect T cells. Indeed, we have already demonstrated that SR 31747A blocks the IL-2/PMA-induced proliferation of purified T cells.[10]

In the lethal septic shock performed in D-Gal-sensitized mice by LPS, we have already shown that SR 31747A was a strong life-preserving agent, when administered before LPS and D-Gal.[12] In that model, we observed that SR 31747A stimulated the LPS-induced IL-10 serum release, and inhibited at the same time the production of TNF-α and IFN-γ. Here, we showed that this sigma ligand was also a potent protector against the lethal effect of SEB and D-galactosamine. IL-2, IL-4 and GM-CSF, which were secreted shortly after SEB injection, were decreased significantly by SR 31747A pretreatment. TNF-α and IL-6, observed to be produced at two different time periods, particularly 12 hr after SEB challenge, were strongly inhibited by pretreatment with the drug. Relating to these two cytokines, TNF-α was already known to play a critical role in the SEB-induced lethal shock, as anti-TNF-α-neutralizing antibodies conferred protection.[16] So, we can postulate that the sigma ligand life-preserving effect may be mediated through the inhibition of TNF-α release. The role of IL-6 in the pathogenesis of the septic shock is so far unclear. However, it should be noted that, in human septic shocks, fatal outcome is always associated with high levels of IL-6,[17] so its role in inducing toxicity has probably been underestimated, and part of the sigma ligand protection, in this model, may be owing to the complete down-regulation of IL-6 production. To our knowledge, it is the first time that IL-6 is observed to be secreted upon challenge with SEB and D-Gal and that both IL-6 and TNF-α are detected in two different time periods.

The life-preserving effect of SR 31747A was observed when the drug was administered before SEB and D-Gal. SR 31747A lost its protective effect when administered simultaneously with or after SEB challenge. These data suggest that the molecule is acting at a very early stage of the process. Because IL-10 was already known to protect mice against SEB-induced toxicity,[13] and was produced shortly after SEB administration, the protective effect of the sigma ligand may be caused by, at least partly, the stimulation of IL-10 production. As IL-10 can be produced by various cells including CD4$^+$ cells,[18] monocytes[19] and B cells,[20] we questioned which cells could be implicated in the enhancement of IL-10 release under SR 31747A treatment. We observed that the increase in IL-10 release was also effective in nude mice, indicating that T cells did not mediate this process. Interestingly, we showed that these deficient mice that are resistant to SEB-induced toxicity, did not display significant levels of IL-6 and TNF-α in their serum. This suggests that the secretion of IL-6 and TNF-α is directly under the dependence of T cells, either these two cytokines are secreted by T cells or their producing cells need T-cell support.[21]

In conclusion, the sigma ligand SR 31747A was highly effective in protecting mice against the lethal effect of SEB. This finding suggests that this agent could be an important candidate for the treatment of septic shocks, besides other reagents such as antibodies to TNF-α[16] or IFN-γ,[14] linomide,[22] cyclosporin A or glucocorticoids. This original compound, devoid of central activity, appears as an innovative therapeutic agent, particularly in patients suffering from inflammatory diseases such as rheumatoid arthritis, where overproduction of TNF-α and IL-6 has been described.[23–28] More generally, the property of SR 31747A to strongly stimulate the production of the natural anti-inflammatory cytokine IL-10, could be of major interest in pathologies in which inflammatory cytokines are involved.

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