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Pre-exposure to the unconditioned or conditioned stimulus does not affect learned immunosuppression in rats

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ABSTRACT

In order to analyze the effects of pre-exposure to either the unconditioned (US) or conditioned stimulus (CS) on learned immunosuppression, we employed an established conditioned taste aversion (CTA) paradigm in rats. In our model, a sweet-tasting drinking solution (saccharin) serves as CS and injection of the immunosuppressive drug cyclosporine A (CsA) is used as US. The conditioned response is reflected by a pronounced CTA and diminished cytokine production by anti-CD3 stimulated splenic T cells. In the present study, experimental animals were exposed either to the US or the CS three times prior to the acquisition phase. On the behavioral level, we found a significantly diminished CTA when animals were pre-exposed to the US or the CS before acquisition. In contrast, US or CS pre-exposure did not affect the behaviorally conditioned suppression of interleukin (IL)-2 production. From the clinical perspective, our data may suggest that conditioning paradigms could be systemically integrated as supportive therapeutic interventions in patients that are already on immunosuppressive therapy or have had previous contact to the gustatory stimulus. Such supportive therapies to pharmacological regimens could not only help to reduce the amount of medication needed and, thus, unwanted toxic side effects, but may also maximize the therapeutic outcome.

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1. Introduction

The intense communication between the central nervous system (CNS) and the peripheral immune system via efferent and afferent pathways is the prerequisite for classical conditioning of immunopharmacological effects (Ader, 2003; Exton et al., 2001; Hadamitzky et al., 2013; Meisel et al., 2005; Schedlowski and Pacheco-Lopez, 2010; Tracey, 2010). Employing a conditioned taste aversion (CTA) paradigm in rats (Garcia et al., 1985), the novel taste of saccharin as conditioned stimulus, (CS) is paired with an injection of the unconditioned stimulus (US), the immunosuppressant cyclosporine A (CsA). In this paradigm, re-exposure to the CS leads to avoidance of the gustatory CS and induces a conditioned suppression of T cell-specific cytokine production (Exton et al., 2001; Pacheco-Lopez et al., 2009; Schedlowski and Pacheco-Lopez, 2010). Findings in experimental animals and humans have demonstrated that this learned immunosuppression can be repeatedly recalled, can be prolonged over time, and is of clinical relevance since it is able to diminish allergic reactions and prolongs the survival time of heterotopically transplanted heart allografts in rats (Albring et al., 2014; Exton et al., 1999; Wirth et al., 2011).

These observations strengthened the considerations that learned immunosuppression could be a useful supportive strategy to pharmacological regimens in clinical situations, with the aim to minimize the amount of drugs and thereby decreasing adverse drug side effects and maximize the therapeutic outcome for the patient's benefit (Ader et al., 2010; Colloca and Benedetti, 2005; Doering and Rief, 2012; Enck et al., 2013; Schedlowski and Pacheco-Lopez, 2010; Schedlowski et al., 2015). In clinical routine, however, the majority of patients will be already on immunosuppressive treatment before participating in an immunosuppressive learning paradigm as a supportive therapy. Thus, the question arises whether and to what extent it would be possible to induce a conditioned immunosuppression in patients that are already on immunosuppressive regimens. Therefore, in order to investigate these so called "US pre-exposure effect" (USPEE) on learned immunosuppression, which was analyzed as, prior presentation of US (LiCl) in the acquisition phase of the conditioning process, leading to a diminished the conditioned response (CTA) (Chang et al., 2007; Meyer et al., 2004; Randich and LoLordo, 1979), we

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exposed male Dark Agouti rats in a first experiment to CsA prior to the start of the conditioning protocol, and subsequently analyzed the conditioned response on the behavioral (CTA) as well as the immunological level (anti-CD3 stimulated IL-2 production). In addition, previous studies showed, that a taste stimulus (CS) which has been repeatedly presented to animals in absence of an aversive US causes an irritation of CTA acquisition leading to a diminished CTA during retrieval, a phenomenon known as latent inhibition (LI) (Albert and Ayres, 1989; Chang et al., 2007; Elkins, 1973; Gaztanaga et al., 2015; Lubow and Moore, 1959). Since it is unclear however, whether LI is also affecting the learned immunosuppressive response, in a second experiment we repeatedly pre-exposed the animals to the CS and subsequently analyzed IL-2 production.

2. Materials and methods

2.1. Animals

Adult male Dark Agouti (DA/HanRj, 200–230 g; Janvier, France) rats were individually caged and kept under an inverse 12-h light/-dark cycle with lights off at 7 am. After arrival, animals were allowed to acclimate to the new surroundings for two weeks before initiation of any experimental procedure. Subsequently, rats were single-housed with *ad libitum* access to food, and tap water was available *ad libitum* until the water deprivation regimen started. The animal facilities and experimental procedures were in accordance with National Institutes of Health and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee (LANUV Düsseldorf, North Rhine-Westphalia).

2.2. Drugs

A stock solution (100 mg/ml) of cyclosporine A (CsA; LC Laboratories, Woburn, USA) containing 900 µl Miglyol (Caelo, Hilden, Germany) and 100 µl ethanol (96%) was diluted with sterile saline (0.9% NaCl, Braun, Melsungen, Germany) to gain the required drug dose of 20 mg/kg body weight in a final injection volume of 1 ml (Hadamitzky et al., 2015).

2.3. Conditioning procedure

Behavioral conditioning was performed as described elsewhere (Exton et al., 2001; Hadamitzky et al., 2015; Pacheco-Lopez et al., 2005, 2009).

Experiment 1 (*US pre-exposure*): Animals were randomly assigned to one of four treatment groups (*CS0; n* = 17, *US; n* = 15, *NPE*; *n* = 16, *PE; n* = 17) and put on a water deprivation regimen (days 1–5, Fig. 1) with two drinking sessions daily of 15 min each. Individual mean water consumption in the morning sessions over these days was taken as baseline level (100%) for "normal" fluid intake. The total amount of liquid consumed per day (morning plus evening session) did not differ between groups (data not shown). During the pre-treatment phase, the conditioned groups *PE* (pre-exposed to the US), *CS0* (control for residual effects of CsA), and the *US*-group (pharmacological control) received intraperitoneal (i.p.) injections of the UCS (20 mg/kg CsA) after the morning drinking session on water deprivation days 3–5. Animals in the NPE-group (the "standard" conditioned group) were injected with saline solution on water deprivation days 3–5 as a control for the handling procedure during the pre-treatment phase. All animals were subsequently conditioned with 0.2% saccharin and 20 mg/kg CsA i.p. on three days with two days between each conditioning trial (acquisition phase). Two days after acquisition, animals of the groups *PE* and *NPE* were re-exposed to the conditioned stimulus without US reinforcement for three consecutive days (retrieval phase), while animals of groups *CS0* and *US* received water instead. In the pharmacological control group (*US*) animals were injected with CsA immediately after the morning water drinking sessions (Fig. 1A and B). During each of the 15 min evening drinking sessions, all animals in all groups received water. To monitor CTA, drinking bottles were weighed before and after each drinking session and fluid intake was assessed. On retrieval day three, animals were sacrificed 1 h after CS-re-exposure, respectively, and spleens were collected for *ex-vivo* immunological analyzes.

Experiment 2 (*CS pre-exposure*): The procedures for Experiment 2 were identical to the ones from Experiment 1. However, the pre-exposure group was replaced by the latent inhibition (*LI*; pre exposed to the CS)-group (*CS0; n* = 18, *US; n* = 18, *NLI; n* = 17, *LI; n* = 18). After water deprivation (days 1–2) the pre-treatment phase started, with the conditioned groups *LI* (pre-exposed to the CS), *CS0* (control for residual effects of CsA), and *US* (pharmacological control) receiving saccharin solution instead of water in the morning drinking session (days 3–5). Animals in the *NLI*-group (the "standard" conditioned group) just received water as a control for the handling procedure during the pre-treatment phase. General conditioning procedures were equal to Experiment 1.

2.4. Cell isolation and stimulation

Splenocytes were isolated by disrupting the spleen with a syringe plunger in a Petri-dish containing HBSS (1× Hank's Balanced Salt Solution, gibco®, Life Technologies™, Darmstadt, Germany). Red blood cells were lysed using diluted BD Pharm Lyse™ lysing solution (BD Pharmingen, Heidelberg, Germany). Splenocytes were washed in cell culture medium (RPMI, 10% FBS, 50 µg/ml gentamycin) and filtered through a 70 µm nylon cell strainer. Cell concentrations were determined with an automatic animal cell counter (Vet abc; Medical Solution, Steinhausen, Switzerland). Splenocytes were adjusted to a final concentration of 5×10^6 -cells/ml and stimulated for 48 h with 1 µg/ml mouse anti-rat CD3 monoclonal antibody (clone: G4.18, BD Pharmingen, Heidelberg, Germany) in order to measure IL-2 cytokine production in the supernatant.

2.5. Cytokine determination

IL-2 cytokine production in sample supernatants was measured using a commercial enzyme linked immunosorbent assay (Quantikine ELISA Rat IL-2; R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Optical density was determined on a Fluostar OPTIMA Microplate Reader (BMG Labtech, Offenbach, Germany) set to 450 nm. Absolute cytokine concentrations were calculated using a log–log curve-fit standard curve. Due to technical reasons some samples were excluded from IL-2 protein analyzes.

2.6. Statistical analyses

Statistical analyses were performed with Sigma Plot (Version 12.3, Systat Software San Jose, CA, USA) and the level of significance was set at $p < 0.05$. Behavioral data (acquisition, retrieval of CTA) were subjected to a two-way analysis of variance (ANOVA) with *Group* (treatment) as one factor, and *Time* (days) as a within-subjects factor. Post hoc comparisons were performed using Bonferroni's corrections. Cytokine production was analyzed using one-way ANOVA with Bonferroni corrections for post hoc comparisons after statistically significant effects in the ANOVA. Cytokine data in Experiments 1 and 2 were combined from two independent experiments and evaluated as mean percentage changes from CS0 control group.

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