In Vivo Administration of a PKA Type I Inhibitor (Rp-8-Br-cAMPS) Restores T-Cell Responses in Retrovirus-Infected Mice

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Abstract: Murine AIDS (MAIDS) is caused by infection with the murine leukemia retrovirus RadLV-Rs and is characterized by T-cell anergy and severe immunodeficiency with increased susceptibility to several experimental opportunistic infections as observed in HIV infection. T cell anergy is associated with an increase of intracellular cAMP level, triggering a multistep pathway involving activation of PKA type I and resulting in inhibition of proximal TCR signaling. We have previously demonstrated that blocking PKA type I using the selective inhibitor Rp-8-Br-cAMPS, restores T-cell function in vitro in MAIDS as well as in HIV infection. In the present report, we investigated the effect of parenteral administration of Rp-8-Br-cAMPS in mice with MAIDS. We show that the compound is not toxic and partially restores the ex vivo proliferative responses to anti-CD3 mAb, but that it has no effect on the lymphadenopathy and splenomegaly characterizing the MAIDS syndrome.

INTRODUCTION

HIV infection is associated with several abnormalities of signal transduction which involve infected as well as non-infected T-cells. Such defects persist even after long periods of viral suppression and hamper restoration of immune responses against pathogens including HIV itself. An increased level of intracytoplasmic cyclic AMP (cAMP) has previously been observed in T cells of HIV infected patients. Increased cAMP participates in the impairment of T-cell receptor (TCR) signaling through a PKA type I/Csk pathway [1, 2] but nuclear translocation of the catalytic subunit of PKA type I has also been observed in MAIDS [8]. Therefore the cAMP/PKA type I pathway could also inhibit more distal steps of TCR signaling through CREM phosphorylation and IL-2 secretion blockade as demonstrated in lupus erythematosus [9].

Treatment of HIV-infected patients with inhibitors of cyclooxygenase 2 (COX-2), which limit exposure of T cells to PGE2 (a cAMP-inducing agent) in the lymphoid organs also allows partial restoration of T cell function [4]. Since other mediators than PGE2 can increase the concentration of intracytoplasmic cAMP, pharmacological agents acting downstream of cAMP (i.e. at the PKA type I level itself) could potentially allow for a more complete restoration of TCR signalling. Rp-8-Br-cAMPS (8-Bromoadenosine-3',5'-monophosphorothioate, Rp- isomer) is an analog of cAMP which occupies cAMP binding sites on the regulatory subunit of PKA type I and prevents dissociation and thus activation of the kinase holoenzyme [6]. In vitro, Rp-8-Br-cAMPS strongly improves the function of T cells isolated from HIV infected patients (even with optimal viral suppression) [1, 4, 7] and from mice with RadLV-Rs murine AIDS (MAIDS) [8]. MAIDS is characterized by a profound anergy of T cells which causes severe opportunistic infection similar to what is observed in HIV infection [10]. In MAIDS, T cells, especially those belonging to the CD4 subset, display a major increase of cAMP concentration which abolishes TCR signaling by a mechanism also operative in human T cells [8, 11, 12]. Once full-blown MAIDS is installed, very few therapeutic approaches have been shown to restore, at least partially, T cell function.

In the present report, we investigated the effect of Rp-8-Br-cAMPS administrated parenterally during 10 days on lymphadenopathy, splenomegaly and T cell anergy which are the main features of MAIDS.

MATERIAL AND METHODS

Mice and Cell Suspension

Male C57BL/6 mice were bred in our facility. Mice were injected twice intraperitoneally (i.p.) at the age of 4 and 5 weeks with 0.25 ml of the cell free viral extract. Age-matched control mice were injected twice i.p. with 0.25 ml phosphate buffered saline (PBS). At different times post infection, mice were killed by CO2 asphyxiation. Peripheral lymph nodes (inguinal, axillary and cervical) were dissociated with syringes to obtain single cell suspensions and passed through a nylon cell strainer, washed three times with complete RPMI 1640 medium and counted on Thoma cytometer after trypan blue exclusion prior to further analysis.
or cell culture. For in vivo experiments, Rp-8-Br-cAMPS 1 mg qd was injected i.p. to the mice during 10 days. In initial experiments aimed at evaluating Rp-8-Br-cAMPS biodistribution, groups of infected and healthy mice were treated by subcutaneous implantation of Alzet osmotic pumps (Cupertino, CA) filled with 10 mg Rp-8-Br-cAMPS dissolved in PBS and set to deliver 0.7 mg/24h. The pumps were implanted 14 days before sacrifice. All studies on mice with MAIDS were conducted under a license delivered to the University of Liege animal facility by the Ministry for Agriculture and with permission from the Local Animal Ethics Committee.

Virus
Viral extract was prepared from lymph nodes of mice injected 2 months earlier with RadLV-Rs as described previously [12]. Lymph nodes were collected, ground in PBS and centrifuged twice at $1.5 \times 10^4$ g for 30 min. This acellular viral extract was stored in liquid nitrogen. XC plaque assay used was as previously described [8, 13] for quantification, and showed that the viral preparation contained $10^3$ particle forming units (PFU) of ecotropic virus/ml.

Reagents and Antibodies
Anti-CD3 mAb (145-2C11) used to activate T cells was from Tebu-bio (Tebu-bio nv, Boechout, Belgium). Rp-8-Br-cAMPS (LA1001) was produced by Biolog (Bremen, Germany) for Lauras AS.

Quantitative Determination of Rp-8-Br-cAMPS Concentration
Suitable sample preparation and HPLC methods were developed for quantitative determination of Rp-8-Br-cAMPS in mice tissues and serum samples that allows evaluation of drug concentrations of in vivo experiments (by BioLog Gmbh, Bremen, Germany for Lauras AS). Calibrations were done with Rp-8-Br-cAMPS and 8-Br-cAMP. Both compounds gave sufficient linearity in a range between 0 ng/ml and 1000 ng/ml. Each mice sample was transferred into a borosilicate micro mortar (1000 μL) followed by addition of 250 μl water. After manual homogenization and addition of 750 μl water the resulting suspension was transferred into 1,5 mL sarstedt-tubes with screw cap. After a minimum period of 4 hours at –70 °C all samples were freeze-dried in a Speed-Vac under oil-pump vacuum overnight. The freeze-dried material was suspended in 1000 μL MeOH/H2O (1:1 v:v) and placed for 15 min in an ultrasonic bath, followed by centrifugation for 15 min (Heraeus; Biofugeprimo; 13000 rpm). 0.85 mL of the supernatant was loaded on an anion exchanger SPE cartridge (Chromafix 400mg SB/Art-Nr.: 731835/Machery-Nagel), washed twice with 2 ml of water and then eluted with 1 ml 0.6 M NaCl. The resulting solution was directly used for HPLC analytics. For complete loading 300 μl of the solute were applied on the 200 μl sample loop. This volume of solute produced reproducible data during calibration of the HPLC method.

Proliferation Assays
Proliferation assays were performed by incubation of 0.1 X 10^6 mixed lymph node cells in a 100 μl volume in 96-well microtiter plates. Activation was achieved by subsequent addition of soluble anti-CD3 mAb (clone 2C11) at a final dilution of 4 μg/ml. Proliferation was analyzed by incubating cells for 72 hours during which [3H]-thymidine (0.4 μCi) was included for the last 4 hours and collected with a cell harvester (Skatron, Sterling, VA, USA) onto glass fiber filters. Incorporated precursor was counted in a scintillation analyzer (Tri-Carb, Packard, Meriden, CT, USA). prior to activation by addition of anti-CD3 antibodies. Rp-8-Br-cAMPS when used in vitro, was added at a final concentration of 1mM, 30 min prior to anti-CD3 mAb. Stimulation indexes were individually determined for each mouse according to the formula : mean cpm in anti-CD3 stimulated cultures/ mean background cpm in unstimulated cultures.

Statistical Analyses
In Figs. (1,2), the data were displayed in Box-and-Whisker plots which illustrate the spread of data groups around their medians. In this type of graph, the box indicates 25 to 75 percentiles whereas whiskeys indicate 2.5 to 97.5 percentiles. Data quoted in the text correspond to means ± sem. Unpaired two-tailed t tests were used for comparisons of different groups of animals. Paired t-tests were used to compare the effect of in vitro exposure to Rp-8-Br-cAMPS vs PBS of the lymphocytes of non-treated infected mice. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) software package.
RESULTS AND DISCUSSION

Initial experiments were performed to evaluate the biodistribution of Rp-8-Br-cAMPS. The concentration of the compound was measured by HPLC on liver, spleen and plasma of the animals implanted with the osmotic pumps set to deliver 0.7 mg Rp-8-Br-cAMPS/24h. As shown in Table 1, Rp-8-Br-cAMPS was found at significant concentration in all the organs analysed demonstrating that the compound was delivered to relevant tissues such as spleen. The toxicity of the Rp-8-Br-cAMPS was also evaluated and as shown in Table 2, there were no unscheduled deaths during the study. Furthermore, there were no clinical signs observed in the mice throughout the time of administration in the different experiments (Table 2). All animals were considered to have achieved satisfactory bodyweight gains through the study. Macroscopic examination at time of sacrifice of the animals did not reveal any abnormalities. Thus, we conclude that Rp-8-Br-cAMPS was well tolerated in control non-infected animals as well as in infected mice.

In a next set of experiments performed with iterative injections of Rp-8-Br-cAMPS during 10 days, we evaluated the effects of the compound in mice with established RadLV-Rs infection. Typically, each experiment encompassed four groups of seven to ten mice (2 groups with mice inoculated with RadLV-Rs eight weeks earlier and 2 groups with age-matched sham-injected controls). Mice were treated either with Rp-8-Br-cAMPS for 10 days (daily intraperitoneal injections of 1mg/mouse) or equivalent sham-injections with 300 μl PBS. At the end of the 10-days injection period, mice were sacrificed. Treatment with Rp-8-Br-cAMPS had no significant effect on the extent of lymphadenopathy and splenomegaly which is typical of RadLV-Rs retroviral infection. Indeed, weights of the lymphoid organs were always similar in infected mice treated with Rp-8-Br-cAMPS and in

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of Mice/Group</th>
<th>Mode of Administration</th>
<th>Rp-8-Br-cAMPS Dose/Mouse</th>
<th>Duration of the Treatment</th>
<th>Death</th>
<th>Clinical Signs of Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 controls non infected</td>
<td>Alzet pumps implanted subcutaneously</td>
<td>10 mg/pump release 0.7mg/day</td>
<td>14 days</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>7 controls non infected</td>
<td>Alzet pumps implanted subcutaneously</td>
<td>10 mg/pump release 0.7mg/day</td>
<td>14 days</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>9 controls non infected</td>
<td>i.p.</td>
<td>30mg/kg/day</td>
<td>10 days</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>7 controls non infected</td>
<td>i.p.</td>
<td>30mg/kg/day</td>
<td>10 days</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>7 controls non infected</td>
<td>i.p.</td>
<td>30mg/kg/day</td>
<td>10 days</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>7 controls non infected</td>
<td>i.p.</td>
<td>30mg/kg/day</td>
<td>10 days</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>6 controls non infected</td>
<td>i.p.</td>
<td>30mg/kg/day</td>
<td>10 days</td>
<td>0</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 1. Biodistribution of the Rp-8-Br-cAMPS in Control and MAIDS Mice After In Vivo Treatment for 14 days with Alzet Pump Loaded with 10 mg of Rp-8-Br-cAMPS. The Given Values for the Rp-8-Br-cAMPS Concentrations are the Mean from Duplicate to Triplicate HPLC Analytics

<table>
<thead>
<tr>
<th>MAIDS/Rp-8-Br-cAMPS (Sodium) in</th>
<th>Concentration (ng/g)</th>
<th>Concentration μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>110 ng/g</td>
<td>0.247 μmol/kg</td>
</tr>
<tr>
<td>Spleen</td>
<td>90 ng/g</td>
<td>0.202 μmol/kg</td>
</tr>
<tr>
<td>Serum</td>
<td>100 ng/ml</td>
<td>0.224 μmol/kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control Mice/Rp-8-Br-cAMPS (Sodium) in</th>
<th>Concentration (ng/g)</th>
<th>Concentration μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>50 ng/g</td>
<td>0.112 μmol/kg</td>
</tr>
<tr>
<td>Spleen</td>
<td>40 ng/g</td>
<td>0.09 μmol/kg</td>
</tr>
<tr>
<td>Serum</td>
<td>90 ng/ml</td>
<td>0.202 μmol/kg</td>
</tr>
</tbody>
</table>

Table 2. Toxicity of the Rp-8-Br-cAMPS In Vivo in MAIDS and Control Mice
infected mice receiving PBS (Fig. 1). Size, and cellularity were also similar in both groups (data not shown). After preparation of cell suspensions from the peripheral lymph nodes of each mouse from the different experimental groups, we measured the proliferative responses to soluble anti-CD3 mAb. The cells were cultured for 72 hours in the presence of anti-CD3 mAb (2C11: 4 μg/ml). During this 72-hour culture period, Rp-8-Br-cAMPS (1 mM) was added to the cells isolated from mice treated with this compound. Administration of Rp-8-Br-cAMPS had no effect on the response to anti-CD3 mAb in non-infected mice (not shown). As expected, in RadLV-Rs retrovirus-infected mice, proliferative responses to anti-CD3 mAb were nearly abolished, with stimulatory indexes (defined as stimulated/non stimulated CPMs) typically around 10% of values reached with the cells of normal mice (Fig. 2). Treatment of non-infected mice with Rp-8-Br-cAMPS did not significantly modify their proliferative responses to the anti-CD3 mAb (not shown). In contrast, i.p. administration of the PKA type I inhibitor Rp-8-Br-cAMPS to RadLV-Rs infected mice strongly increased their responses to the anti-CD3 mAb (31.97 ± 4.21 n=10 vs 5.97 ± 0.88 n=8, p<0.0001). In fact, stimulatory indexes values reached more than 50% of control values in most experiments (Fig. 2). When the cells of infected and treated mice were activated in vitro in the absence of Rp-8-Br-cAMPS, the effect of the treatment was partially lost and became non significant in certain experiments (not shown). When cells from untreated, retrovirus-infected mice were incubated in the presence of Rp-8-Br-cAMPS in vitro, a significant improvement of T cell responses also occurred (12.45 ± 2.02 n=8 vs 5.98 ± 0.88 n=8) (p=0.013) as demonstrated in our previous studies but stimulation index remained much lower than those of infected mice treated with IP injections of the compound (p=0.0014) demonstrating the in vivo impact of PKA type I inhibition on the restoration of proliferative responses in infected mice.

Despite its specific virological features and the preferential tropism of RadLV-Rs for B cells rather than CD4 T cells, MAIDS presents with striking similarities with HIV infection such as polyclonal and sustained immune activation involving CD4 as well as CD8 T cells and leading to the impairment of T cell responses. Indeed, recent evidence suggests that abnormal immune activation rather than direct cytopathic effects of HIV plays a major role in the pathogenesis of HIV infection, at least during its early stages. The PGE2-cAMP-PKA type I pathway is most probably involved in this abnormal activation, as shown by the reduction of activated CD8 CD38 T cells in HIV-infected patients treated with celecoxib [7] and by in vitro data demonstrating partial restoration of T cell responses after incubation with Rp-8-Br-cAMPS [1]. Although COX-2 is indeed overexpressed in HIV infection, it is likely that other soluble factors might play a role in the activation of adenylate cyclase. CCR5 plays a paramount importance in the pathogenesis of HIV infection and this could be partly independent of its function as an entry coreceptor for HIV [14]. Interestingly, HIV coreceptors such as CCR5 and CXCR4 are coupled to protein G and ligand binding activates adenylate cyclase. Gp120 binding to its coreceptor could therefore directly increase cAMP concentration by a PGE2-independent mechanism. It is therefore important to design pharmacological approaches acting downstream of adenylate cyclase activation.

CONCLUSIONS

This is the first report showing an improvement of immune function in a model of retroviral infection after a short course parenteral administration of an inhibitor of PKA type I. Our observations provide proof-of-principle for reversal of retrovirus-induced immunodeficiency by a new class of pharmacological agents directly acting on proximal steps of T cell signalling. Further investigation is warranted to establish if PKA type I blockade also improves antigen-specific immune responses and other immune parameters such as CD4 cytokine secretion and CD8 functions in the infected mice.

ACKNOWLEDGEMENTS

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