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Diazepam leads to enhanced severity of orthopoxvirus infection and immune suppression

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ABSTRACT

Benzodiazepines are drugs widely used as tranquilizers and in various other indications. We treated Balb/c mice with diazepam and infected them with cowpox (CPXV) and vaccinia virus (VACV). Disease index, weight loss and the antibody response were determined. Additionally the influence of different benzodiazepines on the mitogen response of human peripheral blood lymphocytes and spleen cells was tested. Diazepam led to earlier disease onset, prolonged duration of symptoms, higher weight loss and overall disease index in VACV infected mice. CPXV infected mice developed poxviral skin lesions only after drug administration and a significant decrease in the specific antibody response was also observed. Diazepam and alprazolam also inhibited the proliferative response of human lymphocytes/spleen cells *in vitro* but did not show noteworthy apoptotic effects. It is surprising that even a single dose of diazepam has a profound influence on the immune system, sufficient to facilitate symptomatic infectious disease. These data provide first evidence that commonly used drugs like Valium® may augment severity of rare poxvirus infections such as CPXV or monkeypox. As VACV is still used as life vaccine against smallpox there is also a risk of enhanced side effects or possible interference with the success of vaccination.

1. Introduction

Benzodiazepines are a class of substances which function as sedative, anxiolytic, anti-convulsive and muscle relaxing drugs. They act as modulators of the gamma-amino-butyric-acid (GABA) receptor in the central nervous system. Many physiologic functions are modulated by the ionotropic $GABA_A$ receptors, ligand-gated ion channels that are permeable for chloride ions and are the most important inhibitory receptors in the central nervous system (CNS) [1]. Benzodiazepines produce allosteric changes that enhance the action of GABA by increasing the apparent affinity of the receptors and thus the frequency of opening the chloride channels. GABA receptors consist of several subunits, not all of them interacting with benzodiazepines. Classical benzodiazepine binding sites contain β-, γ2- and one of the α-subunits. Sedative effects of benzodiazepines are mediated by α1-subtypes, whereas the α2 and α3-subtypes are responsible for the anxiolytic and anticonvulsant effects [2]. Whereas most classical benzodiazepines show all of the typical effects, more recently introduced substances were developed as selective GABA receptor agonists in order to minimize side effects such as memory impairment and abuse potential [3].

In addition to the receptors in the CNS, peripheral-type benzodiazepine receptors (PBR) were discovered, named after the fact that they bind the benzodiazepine diazepam with relatively high affinity [4]. PBR are multimeric complexes, composed of 18 kDa proteins, and were first detected in kidney tissue, but are present in most peripheral organs in high density. Subcellular fractionation experiments localized PBR in the outer mitochondrial membrane and identified various porphyrins as endogenous ligands [5]. It was speculated that regulation of mitochondrial function could account for the effects of benzodiazepines on cell growth and differentiation, alteration in cardiac actions potentials as well as effects on convulsive thresholds. PBR were associated with increased steroid synthesis, with increased cell proliferation such as in cancer, gliosis, and tissue repair functions [6]. In the CNS, PBR are mainly expressed by activated microglial cells and are considered to play a role in

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neuroinflammatory processes in the pathogenesis of several neurodegenerative disorders [7].

Diazepam (7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one), is one of the best characterized benzodiazepines and therefore on the WHO "list of essential medicines". It has been synthesized in the late fifties and has been on the market under the trade name of Valium® since the mid-60s. It is used in the treatment of anxiety disorders, insomnia, alcohol withdrawal, as emergency medication in epileptic seizures, as premedication for surgical interventions and as part of anesthesia. Pharmacodynamic interactions are observed via metabolism routes involving the cytochrom p450 enzyme (CYP) family. Diazepam shows a high affinity to CYP 3A4 [8] and is metabolized by demethylation and hydroxylation in the human body to nordiazepam, temazepam and oxazepam, all these metabolites showing psychoactive features. The latter two undergo further phase II metabolism steps including glucuronidation.

There are only few indications that benzodiazepines might influence immune functions in humans. Use of midazolam during anesthesia has been reported to influence the interleukin response [9] and an increase of CD8 numbers has been observed upon long term use of benzodiazepines [10]. There are also very limited *in vivo* data on immunomodulating or immunosuppressive effects of benzodiazepines in humans. Indirect evidence arises from a cohort study in HIV positive individuals, where the use of bezodiazepines has been correlated with increased mortality. In multivariate analyses, limited to injection drug users, a positive HIV serostatus, age above 40 years, and using benzodiazepines several times daily were significantly associated with an elevated risk of death [11].

Recently we reported an unusually severe case of cowpox infection [12] in a young male with documented benzodiazepine abuse but showing no signs of immunosuppression [13]. This led us to investigate an effect of diazepam on cowpox infection in an mouse model and to investigate potential drug effects also on human lymphoid cells.

2. Materials and methods

2.1. Virus strains, culture

Vaccinia virus (VACV) laboratory strain WR (Western Reserve), chosen for its known virulence in mice, was obtained from Dr. B. Moss, Bethesda, MD, USA earlier. A human isolate of cowpox virus (CPXV), strain AT/Carynthia/788/07, was grown from local neck infection of a 17-year-old farm girl in Austria [14]. Both viruses were grown on RK13 rabbit kidney cells (ATCC) in roller bottles with enhanced surface (Costar) using DMEM supplemented with 5% FCS. The cells were lysed by freeze thawing, hypotonic medium and brief sonification. The virus supernates were concentrated in a superspeed centrifuge at 20,000 rpm and finally purified over sucrose gradients in a Beckman SW-28 Ultracentrifuge rotor. The resulting virus stocks were diluted in PBS and the virus titer determined by standard plaque assay on RK13 cells in 24-well plates.

2.2. Substances

The benzodiazepines diazepam, nordazepam, oxazepam, flunitrazepam, flurazepam, alprazolam, midazolam, medazepam (Pharmacy of Innsbruck Medical University Hospital, Austria) and lorazepam (Kwizda Pharma, Vienna, AT) were of analytical grade. Diazepam was dissolved in dimethylsulfoxide (DMSO) for the experiments on human PBMC or ethanol for the other experiments. The other drugs tested were dissolved in ethanol. For the experiments they were used as stock solutions of 2.5 mg/ml in solvent and added to the experiments in the indicated dilutions. Phytohemagglutinine (PHA) was purchased from DIFCO Lab., pokeweed mitogen (PWM) from GIBCO Inc., and *Escherichia coli* lipopolysaccharide (LPS) from Sigma–Aldrich.

2.3. Animal experiments

We used female inbred pathogen free Balb/c mice (obtained from Charles River, Germany) which were 6 weeks old and weighed about 16 g. The animals were divided into cages of groups of five per cage and allowed to adjust to the new environment for some days. They then were treated with 10 μg diazepam per gram body weight in a total volume of 100 μl injected via the intraperitoneal route (i.p.). The diazepam was dissolved in a buffer consisting of 10% ethanol and 1% Tween 20 in physiologic saline solution. The same quantity of this buffer without substance was used in the control groups. On the next day the animals were objected to short term anesthesia with ketamin/xylazin (75 and 7.5 μg/g i.p.) and infected with 10 μl of the above viral stocks via the intranasal route (i.n.). The sublethal infectious dose for i.n. inoculation had been determined in pre-experiments (not shown) for the used viral preparations and has been found to be 10^3 plaque forming units (p.f.u.) for VACV and 10^5 p.f.u. for CPXV. These viral doses were chosen for the infection at day 0.

The animals were controlled on a daily basis and the body weights were determined. The disease index (DI) was determined according to a scoring system which has been described earlier [15]. Signs of illness were scored as DI of 0 (normal), 1 (slightly ruffled skin), 2 (clearly ruffled and/or single sided conjunctivitis), 3 (hunched position and/or severe conjunctivitis of both eyes in addition to clear ruffling), 4 (score of 3 combined with difficulty moving/socializing/breathing), and 5 (death). Those animals with a DI of 4 and/or >30% loss of body weight had to be euthanized. At the end of the 14 days observation period the animals were given an anesthesia and blood drawn by heart puncture. Tails were then cut-off immediately from the CPXV infected mice and stained for 5 min with 1% trypan blue in order to visualize necrotic tissue in the poxviral skin lesions.

The work has been evaluated by the local ethics commission and has been approved by the Federal Ministery of Research section for animal experiments (permit BMWF-68.205/0246-II/10b/2008).

2.4. Determination of antibody titer against CPXV

An ELISA system was developed for detection of CPXV specific antibodies in serum. Sucrose purified virus equivalent to 10^6 p.f.u./well was incubated overnight at 4 °C in 50 μl of 0.1 M $NaHCO_4$, pH 9 in 96-well plates (Nunc, Maxisorb®). After removal of the fluid the plates were air dried and exposed on a UV screen for 1 h. Unspecific binding sites were saturated with 0.5% ovalbumin in phosphate buffered saline (PBS, pH 7.4) for 1 h and twofold dilutions of the mouse sera were added in 50 μl of the same buffer containing 0.1% Tween 20 (PBST). Sera were also added to wells without antigen but treated otherwise identically as background controls. After 2 h incubation at room temperature on a shaking platform the plates were washed three times with 200 μl PBST. Bound antibody was detected with peroxidase-labeled rabbit antiserum against whole mouse immunoglobulin (from DAKO, Dk) in the same buffer and conditions as described above. Plates were again washed three times with PBST and one time with substrate buffer (0.1 M acetate, pH 5.5) and finally developed by the addition 50 μl of 5 mM ABTS (2,2-azino-di3-ethylbenzthiazolinsulfonic acid, from SIGMA) and 1 μl/ml of H_2O_2 in the latter buffer. After 1 h the substrate reaction was stopped by the addition of 100 μl of 0.1% sodium azide and the photometric extinction was read in a plate reader at 405 nm. The background values obtained in the wells without virus, which never exceeded 10–20% of the read-