Journal of Neuroimmunology 277 (2014) 105–117

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Intraperitoneal and subcutaneous injections of the TLR9 agonist ODN 1668 in rats: Brain inflammatory responses are related to peripheral IL-6 rather than interferons

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ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form 20 October 2014
Accepted 21 October 2014

Keywords:
Fever
Toll-like receptors
Immune-to-brain signaling
Inflammatory transcription factors
Interferons
Cytokines

ABSTRACT

Subcutaneous or intraperitoneal administration of Toll-like receptor (TLR)-9 agonist, ODN 1668 caused moderate fever and anorexia. In comparison to stimulation of other intracellular TLRs, activation of TLR9 did not result in pronounced peripheral induction of interferons, but rather induced interleukin-6. Expression of cytokines (TNFα, IL-1β) and inducible forms of enzymes for prostaglandin E2 synthesis occurred in the brain, in conjunction with a moderate activation of the transcription factors STAT3 and NF-IL6 in brain endothelial cells. The lack of a septic-like state in ODN 1668-treated rats reinforces the therapeutic value of this drug.

1. Introduction

Highly conserved microbial components called "pathogen-associated molecular patterns" (PAMPs) activate the innate immune system via a family of evolutionary conserved, single-pass, transmembrane proteins termed Toll-like receptors (TLRs). These are coupled to signal transduction pathways that control expression of a variety of inducible immune-response genes (Uematsu and Akira, 2006; Beutler, 2009; Mogensen, 2009; O'Neill et al., 2013). Together with TLRs 3, 7 and 8, TLR9 belongs to the group of intracellular TLRs, which are present in endosomes of dendritic cells, B-cells, macrophages and other cells. They are responsible for intracellular sensing of foreign nucleic acids of viruses and certain bacteria (Blasius and Beutler, 2010). The stimulatory effect of bacterial DNA on TLR9 depends on unmethylated cytosine–phosphate–guanosine (CpG) dinucleotides (Hemmi et al., 2000) and is mimicked by synthetic oligodeoxynucleotides (ODN) containing CpG motifs (Nichani et al., 2004; Kozak et al., 2006).

Agonists of several TLRs have been used for therapeutic interventions, for example as agents for cancer treatment (Adams, 2009), wound healing (Dasu and Isseroff, 2012), or improved elimination of intracellular pathogens (Rajagopal et al., 2010; Guo et al., 2012). For example, TLR9 agonists have been tested and introduced as potent adjuvants in cancer immunotherapy or in combination with conventional chemotherapy (Krieg, 2007; Murad and Clay, 2009; Nierkens et al., 2009). Although the therapeutic use of TLR agonists may be beneficial they can cause undesired side effects (Rosenblatt and de Campos Guidi, 2012). Depending on the route of administration of a given TLR agonist, a marked systemic or local inflammatory response will develop that may affect multiple organs including the brain. Some cell surface TLR agonists even have the capacity to cause a life threatening septic shock-like state and may contribute to organ dysfunction remote from the site where beneficial effects are desired (Schmidt et al., 2007; Al-Saffar et al., 2013; Harden et al., 2014). Although septic shock-like states have not been reported to occur after administration of agonists of intracellular TLRs, repeated epicutaneous application of TLR7 agonists can lead to systemic lupus erythematosus-like autoimmunity in mice

Abbreviations: AP, *area postrema*; COX-2, cyclooxygenase-2; CpG, cytosine–phosphate–guanosine dinucleotides; CVOs, circumventricular organs; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IHC, immunohistochemistry; IκBα, inhibitor kappa B alpha; IL, interleukin; mPGES, microsomal prostaglandin E synthase; NF-IL6, nuclear factor IL6; NFκB, nuclear factor kappa B; ODN, oligodeoxynucleotides; OVLT, *organum vasculosum laminae terminalis*; PAMP, pathogen associated molecular pattern; PBS, phosphate buffered saline; PGE2, prostaglandin E2; RT-PCR, real time polymerase chain reaction; SE, standard error; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; T_{abd}, abdominal temperature; TLR, Toll-like receptor; TNF, tumor necrosis factor; vWF, von Willebrand factor.

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http://dx.doi.org/10.1016/j.jneuroim.2014.10.007
0165-5728/

(Yokogawa et al., 2014). The authors of this study concluded that the skin is the primary organ that allows TLR7 agonists to cause the manifestation of this disease-like state. In this context, it is of interest that subcutaneous administration of a TLR7 agonist causes more potent systemic inflammatory effects than intraperitoneal administration of the agonist. These effects include non-specific illness responses such as fever or anorexia and the expression of inflammatory genes in the hypothalamus, an area of the brain that regulates these symptoms (Damm et al., 2012). These observations with a TLR7 agonist were surprising in so far as intraperitoneal or intra-arterial injections of an agonist of another intracellular TLR (i.e. TLR3) cause much stronger systemic inflammatory responses when compared to the subcutaneous route of administration (Voss et al., 2006).

There are a few studies, in which systemic effects of synthetic TLR9 agonists have been investigated. These studies undertaken in cattle, sheep and mice showed that administration of synthetic CpG DNA (ODNs) induced signs of an acute phase response (Nichani et al., 2004; Kozak et al., 2006) and inflammatory brain activation (Sako et al., 2005). Therapeutic properties and abilities of TLR9 agonists strongly depend on the route of administration (Nierkens et al., 2009; Jarry et al., 2014) with the skin being a major target for therapeutic approaches (Sato et al., 2010; Dasu and Isseroff, 2012).

One central goal of this study was thus to compare sickness and brain inflammatory responses of rats after systemic intraperitoneal (i.p.) or localized subcutaneous (s.c.) treatments with the synthetic CpG oligonucleotide ODN 1668. Our results show that the effects of administering a TLR9 agonist differ to those previously reported for a TLR7 agonist. While s.c. administration of TLR7 agonist strongly activates the interferon (IFN) family of cytokines (Damm et al., 2012), the most likely candidate to cause inflammatory responses within the brain after peripheral administration of a TLR9 agonist is interleukin-6. Activation of distinct intracellular (endosomal) TLRs thus seem to induce similar physiological responses despite activating different inflammatory cytokine patterns.

2. Materials and methods

2.1. Animals

The study was performed in male Wistar rats with body weights in the range of 175–200 g. The experiments were approved by the local ethics committee (ethics approval number GI 18/2 — Nr. 43/2009). After surgery animals were housed individually in a temperature and humidity controlled climatic chamber (Weiss Umwelttechnik GmbH, Typ 10′US/+5 to +40 DU, Germany), which was adjusted to 25 °C and 50% humidity. The rats had constant access to water and powdered standard lab chow ad libitum. The use of special cages with water bottles and food supply dishes placed on balances (AccuScan Instruments, Columbus, OH, USA) allowed continuous monitoring of food and water intake. Artificial lights were on from 7:00 AM to 7:00 PM. Body mass was determined once per day (~9:00 to 9:30 AM). At the same time the food and water reservoirs were re-filled. Animals underwent surgery to implant biotelemetry transmitters (see below) at least one week before the experiment. They were accustomed to the handling procedures within the last three days prior to the experiment.

2.2. Telemetric recording of body temperature, motor activity, food and water intake

Abdominal temperature (T_{abd}) and motor activity of rats were measured using biotelemetry transmitters (model PTD-4000 E-Mitter; Mini-Mitter, Sunriver, OR, USA) implanted into the abdominal cavity at least one week before the experiment. Before the surgery, rats were anesthetized with 100 mg/kg ketamine hydrochloride (Albrecht, Aulendorf, Germany) and 5 mg/kg xylazine (Bayer Vital, Leverkusen, Germany). Analgesic treatment was assured pre- and post-surgery using meloxicam (5 mg/kg, s.c., Boehringer Ingelheim, Ingelheim, Germany). The output from the transmitter (frequency in Hz) was monitored by a receiver placed under each cage (model ER-4000 radio receivers; Mini-Mitter). A data acquisition system (Vital View; Mini-Mitter) was used for automatic control of data collection and analysis. Body temperature was recorded at 5-min intervals. For analysis and graphical documentation, temperature data at time intervals of 15 min were used. Changes in activity were detected by changes in the position of the implanted transmitter over the receiver board. This resulted in a change of the signal strength, which was detected by the receiver and recorded as a pulse of activity. Activity pulses were counted every 5 min and calculated as a cumulative measure of day-time or night-time activity (activity counts per 12 h).

Food intake and drinking behavior were monitored telemetrically using special cages equipped with water bottles and food supply dishes placed on balances, which in turn were connected via a DietScan analyzer to a personal computer (AccuScan Instruments, Columbus, USA). The AccuDiet software package was used to record the data collected for graphical and statistical analysis. Cumulative food and water intake over 5 min were combined into cumulative measures of food and water intake over a 24 hour period. Therefore, the final data represents the cumulative food and water intake in grams per 24 h.

2.3. Drugs and treatments

Lyophilized ODN 1668 (InvivoGen, San Diego, CA, USA) is a synthetic oligonucleotide that contains unmethylated CpG motifs which are present in bacterial DNA. This drug is a specific agonist of TLR9. ODN 1668 was reconstituted in sterile endotoxin-free water at a concentration of 1 mg/ml according to the manufacturer's instructions. Doses of 1 mg/kg or 5 mg/kg were used for i.p. or intra-pouch (see: 2.4) s.c. injections. Control animals were injected with solvent (Ampuva *aqua ad injectionem*; Fresenius, Bad Homburg, Germany). The injection volume was adjusted to 1 ml. All injections were performed at 9:00 AM, 2 h after the start of the "light on" period.

2.4. Experimental protocols

2.4.1. Experiment 1: recording of physiological data

Immediately after placement of a biotelemetry transmitter into the abdominal cavity (see above) a subcutaneous air pouch was formed in about 50% of the rats used for this study. A volume of 20 ml of air, sterilized by passing through a 0.7 μm filter (BD Plastipak; Becton-Dickinson, Heidelberg, Germany), was injected into the subcutaneous tissue of the dorsal midline, caudal to the scapulae. Three or two days prior to the experiment the air pouch was re-inflated with another 10 ml of sterile air [for further details on this model see: Knorr et al., 2008; Knorr et al., 2010; Damm et al., 2012]. One week after ODN 1668 was injected i.p. or into the air pouch (s.c.) at a dose of 1 or 5 mg/kg. Groups of 6–7 rats were used for these experiments. Body mass, food and water intake were determined immediately prior to the respective injection (day 0) to obtain the basal values of these parameters cumulated over the previous 24 h. Further measurements of these parameters were performed 24 h and 48 h after the injections of ODN 1668 or solvent (days 1–2). Body temperature and motor activity were continuously recorded over the experimental period (days 1–2) starting 2 h before the respective injections.

2.4.2. Experiment 2: collection of tissues, plasma, and lavage from the site of localized subcutaneous inflammation

Separate groups of rats (n = 3–5 per group and time point) were treated with ODN 1668 or solvent (see above). According to the results obtained from Experiment 1, only the dose of 5 mg/kg ODN 1668 was used in this experiment. At selected time intervals of 120 min and 360 min, after the injection of ODN 1668 or vehicle, blood from the systemic circulation was taken from all rats. In addition, lavage fluid from