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Inactivated parapoxvirus ovis activates canine blood phagocytes and T lymphocytes

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ABSTRACT

Inactivated parapoxvirus ovis (iPPVO) shows strong immunomodulatory activities in several species and is used in veterinary medicine as an immunostimulatory biological for the prevention and/or treatment of infectious diseases. In this study the immunostimulatory capacity of iPPVO on the innate immune system was investigated *in vitro* by the evaluation of induction of the oxidative burst and modulation of phagocytosis by canine blood leukocytes (polymorphonuclear cells and monocytes) of dogs. In addition, the activation of canine T lymphocytes was also studied. After stimulation with iPPVO the phagocytosis of FITC-labeled *Listeria monocytogenes* was increased in canine blood monocytes and neutrophils. Enhanced burst rates by canine monocytes stimulated with iPPVO were observed and the MHC-II expression on canine CD14+ monocytes was elevated following stimulation with iPPVO compared to the stabiliser control. Canine CD4+ T cells were activated for oligoclonal proliferation in response to iPPVO. This study shows that iPPVO is able to stimulate both phagocytotic and T-cell-dependent immune mechanisms in canine blood leukocytes.

1. Introduction

The family of Poxviridae contains the largest known DNA viruses. Due to their complex genetic structure poxviruses possess various mechanisms for immune evasion by utilizing a variety of genes encoding virulence proteins that counteract the host immune response. Otherwise attenuated poxviruses have immunogenic properties and induce strong innate host immune response (Burgers et al., 2008; Gherardi and Esteban, 2005), probably due to facilitated cellular uptake by macropinocytosis (Mercer and Helenius, 2008). Parapoxvirus ovis (PPVO) shows a restricted host range for sheep and goats with a tropism to the skin and mucous membranes and lack of systemic virus spread (Büttner and Rziha, 2002; McKeever et al., 1988). The virus provides proteins that are viral variants of soluble host immune factors such as an inhibitor of ovine GM-CSF and IL-2 (GIF), viral IL-10, that is very similar to ovine IL-10 and others such as viral vascular endothelial growth factor (VEGF) (Deane et al., 2000; Imlach et al., 2002; Lateef et al., 2003; Scagliarini et al., 2006). The stringent host specificity of parapoxvirus ovis results from capture of host-derived protective immunomodulatory factors (Haig and Fleming, 1999; Haig and McInnes, 2002). For an effective immune response against PPVO, CD4+ T-cells and a humoral immune response seem to be relevant. However, the duration of acquired PPVO-specific immunity is transient commonly resulting in re-infection of the host (Haig and Mercer, 1998; Lloyd et al., 2000).

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The known immunostimulating properties on the innate immune system combined with a weak and transient immune response to the virus makes PPVO an optimal candidate for an immunoactivator. These characteristics are even preserved after inactivation. The immunostimulant Zylexis® is based on the chemically inactivated PPVO (iPPVO) strain D1701 originally isolated from a diseased lamb (Mayr et al., 1981). Before its use as an immunostimulant the strain D1701 had been attenuated by serial cell culture passages which led to substantial genomic changes (Cottone et al., 1998). The attenuated and genetically stable strain D1701 was successfully utilized for the aid in the prevention and treatment of infectious diseases in non-permissive species, especially in several herpes virus infection models (Castrucci et al., 2000; Weber et al., 2003; Ziebell et al., 1997). A major anti-viral effect may result from the strong interferon (IFN)-inducing capacity of PPVO (Büttner et al., 1995).

In this study the immunomodulatory effects of iPPVO were investigated on selected mechanisms of the innate immune response by canine leukocytes. The internalisation and degradation of pathogens in phagocytotic cells form a first barrier of the immune system against pathogens (Serbina et al., 2008; Egan et al., 2008). The engulfment of pathogens by phagocytosis as well as the production of bactericidal active reactive oxygen intermediates (ROI) is primarily achieved by monocytes/macrophages and neutrophils. The latter process is described as oxidative burst because the responsible enzyme complex NADPH oxidase assembles in activated phagocytes and increases consumption of oxygen (Roberts and Camacho, 1967). The impact of iPPVO during early immune responses such as phagocytosis and oxidative burst has not completely been characterized particularly in dogs.

Following uptake and destruction of pathogens by antigen-presenting cells (APC) the pathogens are processed to antigenic peptides and bound to major histocompatibility complex (MHC) molecules for presentation to T cells. In the process the MHC molecules are up-regulated. For MHC-II molecules the class II transactivator (CIITA) is the master regulator of MHC-II transcription that modulates the expression of MHC-II genes (Drozina et al., 2005). In this context it was reported that in PPVO-infected sheep an accumulation of MHC-II+ dendritic cells in skin lesions was found (Haig et al., 1997).

Consequently, the activation of T-cells by iPPVO was a further topic of this investigation. In general, naive T cells that recognize pathogen-derived antigenic peptides/MCH complexes with their T cell receptor become activated and start to proliferate. Following infection with PPVO in sheep, lymphoproliferation in draining lymph nodes and also in efferent lymph was found some days later (Yirrell et al., 1991). In a non-permissive host system such as swine *in vitro* T cell proliferation following stimulation with iPPVO was also observed. In this system the predominant proliferation of T helper cells was induced by iPPVO functioning in a superantigen-like manner independent of antigen processing by APC, demonstrated by a general inhibition of proliferation by an inhibitory anti-MHC-II antibody (Fachinger et al., 2000a).

In this report we demonstrate by several *in vitro* assays for the first time that iPPVO enhances phagocytosis and oxidative burst in canine neutrophils and/or monocytes as well as induces the up-regulation of MHC-II and the proliferation of T helper cells.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS) from *Salmonella* Abortus-equi (Alexis®, Gruenberg, Germany); phorbol-12-myristate-13-acetate (PMA, Alexis®, Gruenberg, Germany); dihydrorhodamine 123 (Invitrogen, Karlsruhe, Germany), fluorescein isothiocyanate (FITC) and cytochalasin D (Sigma–Aldrich, Taufkirchen, Germany) were purchased. Bacteria *Listeria monocytogenes* strain EGD were heat-inactivated (hi) at 60 °C for 1 h and 5×10^8 colony forming units (cfu), incubated with 0.5 mg/ml FITC for 1 h at 37 °C for labelling and afterwards washed three times with PBS.

2.2. Virus

PPVO was chemical inactivated (iPPVO). Pharmaceutical formulation of commercial available iPPVO (Zylexis®) for dogs and cats with at least 230 IFN units of iPPVO strain D1701 per dose, the stabiliser control (polygeline) as well as purified iPPVO (pu iPPVO; without stabiliser) were supplied by Pfizer Animal Health, UK. One dose of pharmaceutical formulation (in the following designated iPPVO) contains 1×10^7 $TCID_{50}$ of PPVO (pre-inactivation) and was resuspended in 1 ml of PBS or medium. For most experiments a 2-fold dilution series of one dose was used starting from 1:4 up to 1:32 (2.5×10^6, 1.25×10^6, 0.63×10^6 and 0.31×10^6 $TCID_{50}$/ml).

2.3. Animals/blood

Blood was obtained from healthy dogs (breeds: beagles, $n = 8$; hybride, $n = 1$) by venepuncture of the anterior cephalic vein into heparinised vacutainer tubes (4 μg/ml lithium-heparin; Kalbe-Labortechnik, Nümbrecht-Elsenroth). Dogs belonged to the College of Veterinary Medicine, University of Leipzig. All procedures were performed in accordance with local legislations and animal welfare legislations.

2.4. Phagocytosis assay

Heparinized blood (150 μl/sample) was stimulated with iPPVO or stabiliser control, LPS (5 μg/ml), or PBS for 20 min at 37 °C in a waterbath. For specific inhibition of phagocytosis Cytochalasin D (5 μg/ml) was co-incubated. 100 μl of FITC-labelled *L. monocytogenes* was added at a multiplicity of infection (MOI) of 10 relative to leukocytes (microscopically counted in Tuerk's reagent in a Neubauer counting chamber) and incubated for a further 15 min at 37 °C. Phagocytosis was stopped by chilling samples on ice for 5 min. For lysis of erythrocytes, blood samples were incubated with lysis buffer (150 mM NH_4Cl, 8 mM $KHCO_3$, 1 mM EDTA; pH 7.0) for 10 min. The lysis was stopped by