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Identification of target cells of a European equine arteritis virus strain in experimentally infected ponies

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ABSTRACT

Currently, little is known on the cellular pathogenesis of equine arteritis virus (EAV). The purpose of the present study was to identify the target cells in ponies experimentally inoculated with EAV *08P178* (EU, clade-1). EAV-target organs (respiratory tissues with associated lymphoid tissues and large intestines), collected at 3 and 7 days post inoculation (dpi) and with virus titers $\geq 10^{5.0}$ $TCID_{50}$/g, were processed with double immunofluorescence staining for the simultaneous detection of EAV N-protein and one of the following cell markers: CD172a (myeloid cells), CD3 (T lymphocytes), IgM (B lymphocytes) and von Willebrand factor (endothelial cells). In the different analyzed organs, 31–58% and 47–63% of the EAV-positive cells were mononuclear leukocytes (mainly $CD172a^+$ followed by $CD3^+$) at 3 and 7 dpi, respectively. EAV-positive endothelial cells were not detected in 3.200 large blood vessels (≥3 endothelial cells/vessel cross section). However, in terminal capillaries (1–2 endothelial cells/vessel cross section) of the different organs, 15–51% of the endothelial cells were EAV-positive. In conclusion, the present study demonstrates that EAV *08P178* (i) has a main tropism for $CD172a^+$ and $CD3^+$ mononuclear leukocytes and (ii) infects a large number of endothelial cells in terminal capillaries. EAV *08P178* infection in capillaries is most probably the cause of an increased vascular permeability leading to leakage of fluid (edema-serous exudate) but not to severe vasculitis and hemorrhages.

1. Introduction

Equine viral arteritis (EVA) is an infectious disease first etiologically defined in 1953, when equine arteritis virus (EAV) was isolated during an abortion outbreak in Ohio (Doll et al., 1968). Since then, serological surveys indicated that the virus is widely distributed in equine populations around the world (Holyoak et al., 2008). Also in Europe, different EVA outbreaks have been reported such as in Switzerland (Bürki and Gerber, 1966), Germany (Golnik et al., 1986; Eichhorn et al., 1995), Spain (Monreal et al., 1995), UK (Wood et al., 1995), Denmark (Larsen et al., 2001), Hungary (Szeredi et al., 2005), France (Hans et al., 2008) and Belgium (Van der Meulen et al., 2001; Gryspeerdt et al., 2009).

Although only one neutralization serotype of EAV has been identified so far (McCollum, 1970; Golnik et al., 1986), a considerable genetic variation among EAV field strains was demonstrated by comparative sequence analysis of ORFs 2–7 (Stadejek et al., 1999; Hornyak et al., 2005). Based on ORF 5 phylogenetic analysis, EAV isolates are clustered into two distinct clades: a North American and a European cluster (Balasuriya et al., 1995; Echeverría et al., 2010). The latter can be further divided

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into two subgroups: EU-1 and EU-2 (Zhang et al., 2010). Distinct EAV isolates appear to vary markedly in their capacity to induce clinical signs (Fukunaga et al., 1981; Balasuriya and MacLachlan, 2004).

The scientific and economic interest on EAV has, in the past, mainly been focused on North American strains (McCollum et al., 1971; Prickett et al., 1972; Timoney, 1984) and only one challenge study has been performed with an Italian isolate (Autorino et al., 1994). After natural and experimental infection with such North American strains, viral antigens were detected, by means of immunohistochemistry staining, in the cytoplasm of cells resembling macrophages, endothelial cells, myometrial and cardiac myocytes, chorionic mesenchymal stromal cells and epithelial cells such as alveolar pneumocytes, enterocytes, adrenal cortical cells, trophoblasts, thymus stroma, renal tubular cells, and male accessory genital gland cells (Jones et al., 1957; Estes and Cheville, 1970; Crawford and Henson, 1972; Del Piero, 2006). However, up till now no studies have been performed to identify EAV infected target cells in target tissues/organs by the use of cell markers.

Recently, we have studied the outcome of an experimental infection in eight seronegative Shetland ponies inoculated with the EAV European strain *08P178* (EU-1 clade) isolated in 2008 from a Belgian field outbreak (Vairo et al., 2012). The ponies were followed until 28 days post inoculation (dpi). After replication in the respiratory tract and associated lymphoid tissues, EAV *08P178* caused a strong cell-associated viremia from 3 to 10 dpi reaching secondary target organs as soon as 3 dpi. Symptoms were mild and consisted of nasal and ocular discharge, fever and lymphadenomegaly. Only one animal manifested a transitory mild scrotal edema. Signs of severe vascular damage such as hemorrhages, petechiae and infarcts were not observed. Histopathologically, intraepithelial, subepithelial and perivascular lymphoplasmocytic infiltration were the most prominent microscopic lesions, which ranged from mild-multifocal to extensive-diffuse (Sabrina Vairo, unpublished results). Congestion was consistently present in the majority of the organs.

The lack of knowledge of the type of target cell in horses infected by European EAV strains and the absence of gross pathological lesions directly associated with vascular damage (*e.g.* hemorrhages) in the experimentally infected ponies (Vairo et al., 2012), stimulated further researches not only to identify the mononuclear cell type(s) involved in the early stages of the pathogenesis (3 and 7 dpi) but also to investigate the possible viral tropism for endothelial cells both in large vessels and terminal capillaries. The present study not only gives better insights in the EAV pathogenesis with a European EAV strain but also allows us to compare our findings with results earlier obtained with North American strains.

2. Materials and methods

2.1. Animals

This study was carried out on samples collected during our previous experimental *in vivo* study (Vairo et al., 2012). Briefly, 8 animals (4 male (M) and 4 female (F)) were oronasally inoculated with 20 ml of phosphate buffered saline (PBS) containing $10^{7.6}$ tissue culture infectious dose 50% endpoint ($TCID_{50}$) of EAV *08P178* (isolated in Belgium (Gryspeerdt et al., 2009), 4th passage on RK13 cells) [GenBank: JN25761]. Using a fenestrated polypropylene 6.8 French catheter of 400 mm length, 5 ml of the virus suspension was administered into each nostril and 10 ml into the mouth. Two additional animals were mock inoculated with 20 ml of PBS. EAV-infected animals were euthanized at different time points post inoculation: M3 and F3 at 3 dpi; M7 and F7 at 7 dpi; M14 and F14 at 14 dpi and M28a and M28b at 28 dpi. The two control animals were euthanized at the end of the experiment (28 dpi of the inoculated animals). This experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC 2009/008).

2.2. Sampling

Immediately after euthanasia, tissue samples were collected as earlier described (Vairo et al., 2012). In the present study, the tissues collected at 3 and 7 dpi were examined because at these time points the most extensive virus replication was present. To maximize the chance to find sufficient infected cells, tissues containing virus titers $\geq 10^{5.0}$ $TCID_{50}$/g were selected for the identification of EAV-positive cells by means of immunofluorescence. This study comprised the following tissues: (i) nasal septum, nasopharynx and tubal nasopharyngeal tonsils, representing the upper respiratory tract (URT); (ii) lungs and bronchial lymph nodes, representing the deep respiratory tract (DRT) and (iii) large intestines (caecum and colon), representing highly susceptible secondary replication sites.

2.3. Immunofluorescence stainings

To quantify and characterize individual EAV-positive cells, a double immunofluorescence staining was performed. Thirty 8 μm-thick cryosections of each tissue mentioned above were cut and fixed in methanol at −20 °C for 20 min. For each tissue, ten cryosections were stained for each cell surface marker separately. In the first step, optimal dilutions of mAb DH59B (VMRD), UC F6G-3 (California University, Davis, USA) or 1.9/3.2 (VMRD) were used as markers for $CD172a^+$ cells (myeloid lineage), $CD3^+$ cells (pan T lymphocytes) and IgM^+ cells (B lymphocytes), respectively. Afterwards, slides were incubated with Texas Red®-labeled goat anti-mouse IgG antibodies (Molecular Probes). In the second step, EAV nucleocapsid (N) protein was visualized with mAb 17D3 (VMRD), directly labeled with Zenon® Alexa Fluor® 488 Mouse IgG1 Labeling Kit (Molecular Probes), according to the manufacturer's instructions. As negative control, 8 μm-thick cryosections of nasopharynx, lungs and colon of mock-inoculated animals were stained following the aforementioned protocol. In addition, ten extra sections of EAV inoculated animals were incubated with $CD172a^+$ as primary antibody and Texas Red®-labeled goat anti-mouse IgG antibodies as secondary antibody. In a second step, the slides were further incubated with an irrelevant IgG1 directly labeled