Introduction

The major virulence feature of every intracellular infectious agent (viruses, intracellular bacteria) is the way it moves from one host cell to another, what results in the spread of the infection. These pathogens have evolved a broad spectrum of mechanisms to exploit the host cell, among them a way to manipulate one of the three major cytoskeletal systems – actin microfilaments. Non-related invasive bacteria, e.g. *Listeria* sp., *Shigella* sp., *Rickettsia* sp., *Burkholderia* sp., and *Mycobacterium* sp. share the ability to infect non-phagocytic cells. They independently developed similar actin-based mechanisms named as actin tails or "comet tails" that are essential for inter-cellular propagation of the infection and avoidance of the innate and adaptive immune responses (Tilney and Portnoy, 1989; Cudmore et al., 1996; Cossart and Sansonetti, 2004; Gouin et al., 2005; Hybiske and Stephens, 2008). As obligate intracellular parasites, the viruses also evolved a variety of interactions with host cell actin upon entry, replication and egress. For example, Ebola virus requires the actin filaments for its uptake, and Influenza A virus nucleoprotein have been found to co-localize with the actin in cytoplasm of infected cell (Cudmore et al., 1997; Döhner and Sodeik, 2004; Radtke et al., 2006). Moreover, the microfilaments are used for a viral transcription by Hu- man respiratory syncytial virus or may be incorporated in virions during morphogenesis of Rabies virus and Human immunodeficiency virus 1. Additionally, the latter virus needs actin for the export of unspliced genomic RNAs, budding and transmission to the uninfected cells (Cudmore et al., 1997; Döhner and Sodeik, 2004; Radtke et al., 2006). However, none of the described interactions is as special as Vaccinia virus (VACV) intracellular motion by means of the actin microfilaments (Highley and Way, 1997).

VACV is the best known prototype member of the genus *Orthopoxvirus*, the family *Poxviridae*. It is one of the largest and most complex dsDNA viruses used as a live attenuated vaccine against smallpox caused by Variola virus (Fenner et al., 1989). During VACV replication cycle occurring entirely...
in the cytoplasm, several different morphological forms are produced. Among them two infectious particles can be distinguished: mature virion (MV) constituting the majority of viral progeny and extracellular virion (EV) released outside the cell (Smith et al., 2002; Moss, 2006). Some EVs remain on the cell surface and are called cell-associated enveloped virions (CEVs). CEV plays a role in the spread of virions to the adjacent cells, while EV is responsible for the spread of virions to longer distances in vitro and probably also in vivo (Cudmore et al., 1996; Smith et al., 2002; Smith et al., 2003).

First reports about VACV abilities to induce a formation of protrusions similar to microvilli tipped with viral particles were reported in 1970s (Stokes, 1976). Following research brought evidence that formation of the structures composed of actin and its cross-linking proteins is associated with the virus (Hiller et al., 1979, 1981; Krempien et al., 1981).

Present knowledge indicates that VACV exploits actin filaments during the late stage of its replicative cycle, while spreading directly from cell to cell (Radtke et al., 2006). The fusion of the wrapped virion (WV) and the cell membrane leads to the release of structural viral proteins B5R and A36R that remain on the cell membrane underneath CEV. B5R protein activates Src-family kinases that phosphorylate tyrosine cytoplasmatic domains of A36R protein (Frischknecht et al., 1999; Newsome et al., 2004). This event results in the release of kinesin-1 from A36R protein and in the activation of actin polymerization enabling CEV transmission to the neighboring cells through the formation of actin tails (Smith et al., 2003). It can be independently induced by Src- and Abi-family kinases and the latter induce also release of CEV from the infected cell (Reeves et al., 2005; Radtke et al., 2006).

ECTV, the causative agent of mousepox, is closely related to VACV, but as opposed to VACV it has a very narrow host range (Buller and Palumbo, 1991). Only H-2^k, H-2^d, and H-2^e mice are sensitive to ECTV lethal infection, but not H-2^a mice (Buller et al., 1986). Moreover, ECTV genome analysis showed its location on a separate branch of the phylogenetic tree in relation to other orthopoxviruses (Chen et al., 2003; Gubser et al., 2004). However, the terminal regions of the ECTV genome have higher degree of similarity to VACV than to other poxviruses (Ribas et al., 2003).

The aim of this work was to document the changes induced by ECTV infection in the morphology and actin network of permissive cells of different origin. These findings may enable us to evaluate the involvement of ECTV in actin-based mechanisms for low-range spread.

Materials and Methods

Cell lines. HeLa cells and Vero cells were grown in DMEM (Invitrogen) supplemented with 7% FBS (Invitrogen). BALB/3T3 clone A31 cells were grown in DMEM supplemented with 4 mmol/l L-glutamine and 10% calf serum (ATCC). All cell lines were incubated at 37°C in 4.5% CO₂, humidified atmosphere.

Virus. A highly virulent Moscow strain of ECTV (ECTV-MOS; ATCC 1374) was propagated in Vero cells. Its infectivity was determined by plaque assay on Vero cells (Reeves et al., 2005). Cell infection was carried out in DMEM with 1% of serum at MOI = 1. For mice experiments, 6-week-old BALB/c (H-2^k) mice were foot-pad infected with ECTV-MOS. At 12 days p.i. the spleens were aseptically removed and prepared for transmission electron microscopy (TEM) (Spohr de Faundez et al., 1995). Ultrathin spleen sections were stained with uranyl acetate along with lead citrate and observed under a Jeol 100C TEM microscope (Japan).

Antiserum. Rabbit polyclonal antiserum was raised against complete ECTV-MOS particles. Briefly, after five injections of antigen, the rabbits were bled and the immune serum was obtained. Affinity chromatography purified polyclonal anti ECTV antibody was FITC-conjugated and used in immunofluorescence assays.

Immunofluorescence microscopy. Cell monolayers were grown on coverslips to reach 70–80% confluence and infected with ECTV as described above. At 19 hrs p.i. the infected cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.5% Triton-X and processed according to Mitchison Lab (Harvard Medical School) procedure (http://mitchison.med.harvard.edu/protocols/gen1.html). F-actin was stained with Alexa Fluor-633- (Invitrogen) or TRITC-phalloidin (Sigma). For visualization of DNA, Hoechst 33342 (Calbiochem) was used (1 μg/ml). Images were recorded using Olympus BX60 fluorescence microscope equipped with Color View III cooled CCD camera (100× immersion objective) or with Leica TCS SP2 confocal laser scanning microscope (63× immersion objective, digital zoom 2×). Subsequently, images were processed using the Cell®F software (Olympus) and Adobe Photoshop. The presented results were selected from triplicate experiments.

Immunoblot analysis. Vero cells were infected with ECTV at MOI = 1. At 8 or 24 hrs p.i. the cells were lysed and the proteins were extracted with Mammalian Cell Lysis Kit-1 (Sigma). The protein concentration of each sample was determined using QuantiPro® BCA Assay Kit (Sigma). An equal amount of protein for each sample was loaded onto 12% polyacrylamide gel. After PAGE, the proteins were electroblotted onto PVDF Westran Clear Signal (Whatman). To detect β-actin, blots were probed with mouse monoclonal anti-β-actin antibody (Sigma) followed by rabbit anti-mouse immunoglobulin conjugated with peroxidase (Sigma) and Chemiluminescent Peroxidase Substrate-1 (Sigma). The samples were analyzed by KODAK IMAGE STATION 4000MM (Kodak MI SE Program).

Results and Discussion

The cytoskeletal architecture plays a significant role in the spread of virus infection from one permissive cell to another. Moreover, viral cytoplasmic transport and their molecular interactions with cytoskeleton of the infected host are critical for effective innate and adaptive immune
responses. The aim of our study was to gain a better insight into ECTV interactions with the cytoskeleton of permissive cells of different origin: heterologous – human and monkey cells and homologous – cells derived from BALB/c mouse strain highly susceptible to ECTV. Duration of infection (19 hrs) was chosen on the basis of earlier observations of VACV, myxoma virus and ECTV replication cycle (Sodeik et al., 1993; Niemialtowski et al., 1994; Reeves et al., 2005; Duteyrat et al., 2006). At 19 hrs p.i., all viral morphological forms MVs, WVs, EVs, and CEVs should be present and changes in the cytoskeleton should be well-defined.

The mice experiments showed that virus preparation used in our research was able to induce the infectious mousepox in BALB/c (H-2\textsuperscript{d}) mice that displayed all the classical signs of the disease: hunched posture, rough hair coat, conjunctivitis, dermal pox lesions, swelling of the face and extremities (Fenner, 1981). Additionally, TEM observations of ultrathin sections prepared from infected spleens revealed the presence of viral particles with typical brick-shaped morphology (Fig. 1).

We observed that ECTV like VACV (Radtke et al., 2006) induced alterations in the microfilament organization of the infected cells. These changes were best characterized by an abnormal morphology, e.g. shrunken, rounded and clumped appearance (Figs. 2, 4, 5) or by a formation of syncytia (Fig. 3). There was also a noticeable loss of stress fibers and increase in actin content of the cell cortex (Figs. 2, 3, 5) as compared to the neighboring non-infected cells. This may be the result of a shift of actin to the cell periphery, because additional actin is needed for the generation of protrusions. The infected cells produced either individual, long and thick projections similar to filopodia (Figs. 3, 4) or several kinds of small actin-rich microvilli resulting in the stelliform appearance of the cell (Fig. 2). Among them were included the specialized microvilli closely resembling VACV-associated actin tails (Cudmore et al., 1996; Smith et al., 2003; Radtke et al., 2006). These structures are being formed in different cell lines. In our experiments we observed them in human HeLa cells (Fig. 2) and murine BALB/3T3 cells (Fig. 5). In the second case, the tails were well-defined, but not very abundant as compared to the numerous tails described for VACV (Zhang et al., 2000). It appeared that they contributed to the cell-to-cell spread (Fig. 5). This mechanism of viral transmission may seem ineffective, when compared to the cell lysis that is believed to be the main mechanism of virus spread. However, the virus spread mechanism could be different in vivo as proven for neurotropic herpesviruses. The virus release by cell lysis was not required for their dissemination and pathology (Heffner et al., 1993; Mulder et al., 1996). Our study revealed for the first time the importance of actin polymerization in the spread of ECTV from cell to cell in vitro, what suggested that this type of infectious particles motion could be typical for orthopoxviruses. Among members of other virus families similar actin-rich microvilli have been observed on the surfaces of cells infected with Murine mammary tumor virus and Frog virus 3 (Cudmore et al., 1997; Smith and Enquist, 2002). It is not known yet, if these protrusions also take part in the dynamic spread of these viruses, but they clearly play an important role in the budding of these viruses.

We noticed more prominent structures than actin tails in HeLa and Vero cell lines (Figs. 3, 4) containing numerous
virions in contrast to a tail that propelled only one virus particle to its tip. These structures resembled projections that have been described as filopodia for VACV, strain MVA or as "cytoplasmic corridors" for myxoma virus (Gallego-Gomez et al., 2003; Duteyrat et al., 2006). Similar actin-containing projections have been also found to be induced by several herpesviruses (Favoreel et al., 2007). Above-mentioned structures seemed to contribute to the direct spread of virions into adjacent cells without being exposed to the external microenvironment. This mechanism along with macropinocytosis and apoptotic mimicry that VACV uses to enter different cell types (Mercer and Helenius, 2008), could be another example of the poxviruses strategy for an effective escape from cellular and molecular components of the immune system.

To investigate whether all the abovementioned prominent changes in the actin cytoskeleton affected the total level of β-actin in cells during ECTV replicative cycle, cell lysates of mock-infected and ECTV-infected (8 and 24 hrs p.i.) Vero cells were prepared and used in immunoblot analysis. Actin-specific bands of 43 K were detectable during the entire observation period for both infected and mock-infected cells (Fig. 6a) The amount of actin in infected cells was heavily reduced at 24 hrs p.i., what was quantified by densitometry (Fig. 6b) showing an over 5-fold decrease compared to the mock-infected cells. This finding is quite unusual as actin is considered to be a housekeeping gene and protein and is widely used as

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**Fig. 2**

**Immunofluorescence of ECTV-infected HeLa cells**

Staining for actin (a), ECTV (b), and cellular nuclei and viral DNA – extranuclear replication centers are indicated by an asterisk (c). Merged images – red box is shown in expanded scale. White arrow indicates ECTV virion on the tip of actin tail (d).
an immunoblot loading control (Guerra et al., 2003; Kuhn et al., 2005). However, further investigation is needed, but these results suggested reconsideration of actin as a reference protein/gene for the experiments with ECTV.

In conclusion, we showed that the actin network of cells contributed to the plasticity of cells in non-infected or infected state. Our experiments demonstrated for the first time that ECTV infection induced actin rearrangements and production of dynamic protrusions in the cell lines of

Fig. 3

Immunofluorescence of ECTV-infected HeLa cells
Staining for actin (a), ECTV (b), and cellular nuclei and viral DNA (c). Merged image – white arrow indicates actin-rich projection containing several virions (d).

Fig. 4

Immunofluorescence of ECTV-infected Vero cells
Staining for ECTV, white arrows indicate filopodia containing several ECTV virions.
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**Fig. 5**
Confocal microscopy of ECTV-infected BALB/3T3 cells
Staining for actin (a) and ECTV (b). Merged images – actin tails on cell surface (white arrows), virions (yellow arrows) and a viral particle delivered via actin tail to neighboring cell (blue arrow) (c).

**Fig. 6**
Effect of ECTV infection on β-actin content in Vero cells
Immunoblot analysis (a) and its quantitative evaluation (b). The positions and size of marker proteins are indicated on the left (a). The bars indicate standard deviation (b).

different origin, but the effect was cell-line dependent, e.g. virus-containing filopodia in Vero in contrast to the actin tails in BALB/3T3 cells. These results suggest that the kind of developed protrusion is connected with some unique host factors that remain to be identified. We hope that the results presented here will set a base for future experimental studies exploring not only the pathologic manifestation of ECTV infection, but also the regulation of in-cell signaling.

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**References**


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