Entry of Ebola Virus is an Asynchronous Process

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Ebola virus (EBOV) is responsible for a severe fever with a high mortality rate. The diverse nature of the attachment of the virus to the cell surface, the initial step of virus entry, raises questions concerning the kinetics of the virus internalization process. We investigated EBOV entry kinetics using the activity of a particular monoclonal antibody that neutralizes virus infectivity. We demonstrate that inoculation of cells with EBOV results in an asynchronous entry process, as revealed by the ability of the virus to remain in a cell-bound state for an extended period of time before it is internalized.

Keywords. Ebola virus; family Filoviridae; glycoprotein GP; virus attachment; virus neutralizing antibody; virus entry kinetics.

Ebola virus (EBOV) causes a severe form of hemorrhagic fever in humans and nonhuman primates [1]. EBOV is member of the family Filoviridae, a group of enveloped, nonsegmented, negative-strand RNA viruses [2]. The EBOV genome is about 19 kb long, containing 7 linearly arranged genes that program the expression of 7 structural and 3 nonstructural proteins [3–6]. The surface glycoprotein GP mediates virus attachment and entry into host cells. [7–10]. The GP molecule is highly glycosylated, with both N- and O-linked glycans. GP is cleaved by the cellular protease furin to generate a disulfide-linked GP1-GP2 heterodimer [11]. The virion spikes are assembled as a trimer of the GP1-GP2 molecules. It is generally accepted that viral cell entry occurs through virus attachment to the cell surface, followed by endocytosis (macropinocytosis or clathrin-mediated endocytosis; reviewed elsewhere [12]). After internalization, the GP undergo a proteolytic digestion at acid pH by cellular proteases cathepsin B and cathepsin L [7], leading to subsequent liberation of the receptor binding domain that interacts with an intrareceptor, the Niemann Pick C1 protein (NPC1), an endosomal/lysosomal transporter of cholesterol [13]. Conformational rearrangements associated with the interaction between NPC1 and the cleaved GP are believed to result in exposure of a fusion loop that facilitates membrane fusion and the liberation of the viral nucleocapsid into the cell cytoplasm [14]. A significant part of surface GP is released from virus-infected cells owing to cleavage by the cellular metalloprotease TACE/ADAM17, giving rise to a soluble form of surface GP known as shed GP, which seems to play an important role in virus pathogenicity [15, 16]. The attachment of virions to the cell surface relies on a wide range of lectins (e.g., dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin [DC-SIGN], DC-SIGN homologue [DC-SIGN-R], Liver and lymph node Sinusoidal Endothelial C Type Lectin [LSECtin], and folate receptor α) that recognize EBOV GP, and, in particular, its atypical and high glycosylation pattern [17–19]. There is also evidence for GP-nonspecific attachment of EBOV via phosphatidylserine molecules present in the virion membrane and cell surface T cell/transmembrane, immunoglobulin, and mucin (TIM) and Tyro3, Axl, Mer (TAM) family receptor [20–22].

In the current study, we investigated EBOV entry kinetics using the activity of a particular monoclonal antibody (mAb) that neutralizes virus infectivity. We demonstrated that inoculation of cells with EBOV results in an asynchronous entry process, as revealed by the ability of the virus to remain in a cell-bound state for an extended period of time before it is internalized.
MATERIALS AND METHODS

Viruses, Cells, and Reagents
Recombinant Zaire ebolavirus, strain Mayinga, expressing green fluorescent protein (EBOV-GFP) was generated as described elsewhere [23] and used for infection of Vero E6, Huh7, and EpoNi cells, a bat (Epomops franqueti) kidney-derived cell line [24]. Cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS). Experiments using EBOV were performed in the BS14 INSERM Laboratory Jean Mérieux.

Reynard et al [25] have presented data on a panel of 87 mAbs directed against Ebola virus GP. One mAB 3327, displayed strong virus-neutralizing activity (O. Reynard and V. Volchkov, unpublished data). This mAb was produced in CD hybridoma medium (Life Technologies) and purified with protein G sepharose affinity chromatography (GE Healthcare).

Kinetic Assay and Flow Cytometry
EBOV-GFP was used to inoculate Vero E6, Huh7, or EpoNi cells at a multiplicity of infection of 0.5 in 24-well plates, using 0.3 mL of inoculum. After incubation for 1 hour at 37°C, virus was removed and cells were washed twice with 1 mL of DMEM before the addition of DMEM–3% FCS. At time 0, cells were inoculated with a mix of the virus and mAb 3327 for 48 hours. At 1, 2, 4, and 6 hours after inoculation, DMEM–3% FCS was replaced by medium containing 10 µg/mL of mAb 3327, leading to neutralization of cell-bound virions. Cells were trypsinized and analyzed using flow cytometry performed with a Beckman Gallios instrument 48 hours after infection. All experiments were performed in triplicate.

Plaque Assay
Subconfluent Vero E6 cells in 6-well plates were infected with serial dilutions of EBOV-GFP. After 1-hour incubation, the inoculum was removed, and the cells were overlaid with 0.4% agar containing DMEM–3% FCS. Plates were incubated for 72 hours at 37°C and then subjected to imaging under a Nikon UV microscope (Multizoom AZ1100). Plaque size (fluorescent area) was measured using ImageJ software (version 1.46R, National Institutes of Health) [26].

RESULTS AND DISCUSSION

EBOV entry is a multistep process that begins with attachment of virus particles to the cell surface. Several cellular factors—DC-SIGN/liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin, LSECtin, human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin, β-integrins, and Tyro3 family receptors—have been proposed to play a role in virus attachment and could explain the pantropic character of EBOV infection [12]. The diverse nature of the initial attachment step raises questions concerning the kinetics of the virus internalization process. To address these questions, we used a recently described neutralizing antibody, referred to here as mAb 3327, which neutralizes virus particles at a late step of virus entry, presumably before membrane fusion but after GP cleavage by cellular cathepsins and interaction with NPC1 (O. Reynard and V. Volchkov, unpublished data). This mode of action implies that the antibody is able to bind virus particles and is then internalized along with the virion. In this sense, virions that are internalized before antibody binding are no longer susceptible to its neutralization action. We were thus able to monitor the kinetics of the viral internalization process by adding this antibody at different time points after the removal of the viral inoculum.

In the experiment shown in Figure 1, mAb 3327 was added to Vero E6 cells either simultaneous with the virus at time 0 or at different time points up to 6 hours after inoculation with a recombinant EBOV expressing GFP (EBOV-GFP). After 48 hours of growth in medium containing 10 µg/mL mAb 3327, the number of infected cells was measured by counting GFP-positive cells using flow cytometry. In this manner, we were able to evaluate the number of cells infected at the different time points and before the administration of neutralizing antibodies. Culturing of infected cells in the presence of mAb allows us to avoid possible difficulties in interpreting the results related to release of virus from the cells and subsequent second-round infection. Of note, at 6 hours after inoculation in this assay setup, we consider that we can no longer discriminate between infection coming from the initial inoculation and that arising from newly

Figure 1. Kinetics of Ebola virus (EBOV) entry. Kinetics of EBOV entry were analyzed using the ability of monoclonal antibody (mAb) 3327 to neutralize virus attached to the cell surface. Three cell lines were used (EpoNi, Huh7, and Vero E6), and mAb 3327 was added to the cells at different times up to 6 hours after inoculation with EBOV expressing green fluorescent protein (GFP). After 48 hours of growth in medium containing 10 µg/mL mAb 3327, the number of infected cells was measured by counting GFP-positive cells, using flow cytometry. Thus, the number of cells infected at the different time points and before the administration of neutralizing antibodies was evaluated. All results are shown normalized to levels seen for time point 6 hours after infection for each cell type. Data represent mean values from 3 independent experiments.
released virions. To compare cell lines, all results are shown normalized to levels seen for time point 6 hours after infection.

As shown in Figure 1, for certain cell lines a surprising number of EBOV particles seem to be not yet internalized at 4 hours. It is also apparent that internalization seems to be a clearly asynchronous process. As measured by the entry kinetics shown in Figure 1, internalization in Huh7 cells seems to require even longer than in Vero E6 cells. In sharp contrast, for a cell line derived from the kidney of the bat *E. franqueti*, a suggested natural reservoir of EBOV, the virus entry process is extremely fast, and most particles are internalized in a more synchronized manner. These results show that, in this setup, diverse cell types or cells of different origins clearly differ in terms of the kinetics of EBOV entry, in particular, virus internalization. The observed differences in entry kinetics are most likely multifactorial and can obviously implicate both the density and

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**Figure 2.** Heterogeneity in Ebola virus (EBOV) plaque size. EBOV plaques are unusually heterogeneous in size on agar-overlaid Vero E6 cells. A, Two examples of small and large plaques formed by EBOV-GFP. B, Six plaque-isolated viral clones, either small (S) (<100 µm) or large (L) (>800 µm) in diameter at passage 0, were amplified in Vero E6 cells and then subjected to analysis of plaque size. Left, Variation in plaque size for this second passage is shown as a mean and standard deviation for each selected viral clone. Right, Variation in plaque size for initially selected groups of either small- or large-plaque viral isolates is shown as the range of the mean (boxes), the median (middle line), and the mean of their standard deviation (whiskers). C, A third round of selection of EBOV viral clones for plaque size was performed for a number of both large- and small-plaque clones, and data are shown as described above. LL and SS represent clones for which a large- or small-plaque clone, respectively, was selected at both passage 0 and passage 1; LS, clones for which a large-plaque clone was selected at passage 0 and a small-plaque clone at passage 1; and SL, clones for which a small-plaque clone was selected at passage 0 and a large-plaque clone at passage 1. Right, No significant differences in plaque size distribution were found for any group.
specificity of surface attachment molecules or the propensity of a cell type for macropinocytosis. Importantly, the data presented here clearly indicate that adherence of virions at 37°C is followed by an asynchronous process, with cells internalizing infectious particles from within a few minutes after inoculation to at least several hours later. That some particles can be very rapidly internalized after attachment to target cells is also illustrated by data in Figure 1 showing that, even for cells inoculated in the presence of antibody (time point 0), a number of particles are internalized before mAb 3327 interacts to neutralize the virus. In this regard, our observations are consistent with those of several studies showing that at least some particles are quickly internalized at 37°C after an adhesion step at 4°C [27, 28].

It is interesting to speculate what is happening with viral particles after they attach to cells but are not yet internalized. It is plausible that, with a low concentration of attachment molecules on the cell surface and/or low affinity, viral particles can temporally detach from the cell surface before reattaching, in a mechanism that thus would allow a “scanning” of the cell surface for a better place to stably attach. Alternatively, this may also allow the virus to “change” target if the initial cell is not suitable for entry in some way. It could also allow virus to be delivered within the host from the initial site to a site of its replication, and such a mechanism might be important at the early stage of infection. Such transinfection as a way to spread within the host has been demonstrated for human immunodeficiency virus [29] and more recently for Nipah virus [30]. Therefore, a delay in virus internalization might not be as detrimental for the virus as it seems at first glance, and it might even provide some important advantages.

This phenomenon probably also highlights the need for the viral particles to reach an area on the cell surface with sufficient concentration of adhesion factors to promote particle stabilization and internalization, and this sum of adhesion factors acting for particle stabilization is probably cell type specific. Of note, Vero E6 cells are not EBOV primary target cells, even though they are widely used to grow EBOV and many other viruses owing to defects in interferon production. In future experiments it will be important to also study virus entry kinetics using primary virus targets, and this work is currently ongoing.

The asynchronous entry of EBOV into target cells could obviously be expected to have repercussions for the subsequent initiation of viral replication. Indeed, in cell culture, EBOV plaques were unusually heterogeneous in size on agar-overlaid Vero E6 cells. The differences in plaque size could therefore be explained by early or late internalization after attachment. If this hypothesis is true, one would not expect plaque size to be stable during clonal selection of plaques of different sizes. To test this idea, we analyzed the plaque size of EBOV-GFP and isolated several clones of plaques displaying either small or large diameters (Figure 2A). These clones were grown for 48 hours in Vero E6 cells and then analyzed again for plaque size. As shown in Figure 2B, plaques from all clones displayed a wide variety of sizes, as seen by the standard deviation between groups (≥35 plaques measured for each clone).

When a second cloning step was performed, similar results were obtained, except for 2 clones that were found to induce permanently smaller diameter plaques (clones SS1 and SS6, with a small mean diameter and small standard deviation). It will be interesting to determine whether these 2 clones have any specific genotype with the appearance of stable mutations that might limit their growth. The appearance and subsequent selection of plaques showing a particular and specific phenotype (large plaque size) is known to be associated with EBOV/8U (variant containing 8 uridine residues at the GP gene editing site), which were selected during plaque purification [3–5]. The EBOV/8U variant was shown to have significant growth advantages over wild-type EBOV (EBOV/7U) in cell cultures. Although other factors (eg, cell cycle stage at the moment of virus entry) may also have an effect on EBOV plaque size heterogeneity, the data provided support the idea that the asynchronous and apparently substantially delayed entry of EBOV into target cells could explain this phenomenon.

In conclusion, in this study we demonstrated that EBOV entry is an asynchronous process, as revealed by the property of the virus to remain in a cell-bound state for an extended period of time before it is internalized and then induce virus replication. Future studies will have to reveal the full significance of this delay in virus internalization during EBOV replication and the repercussions for this phenomenon within an infected host organism.

Notes

Acknowledgments. All experiments involving live EBOV were carried out in the INSERM BSL4 Laboratory Jean Mérieux in Lyon, France. We are grateful to staff of the SFR BioSciences cytometry platform of and to the INSERM BSL4 Jean Mérieux team members for technical assistance and support. We also thank Drs Philip Lawrence, Mathieu Mateo, and Xavier Carnec, International Centre for Research in Infectiology, Lyon, for helpful discussion and editing of the manuscript.

Financial support. This work was supported by INSERM, Agence Nationale de la Recherche (grant ANR-07-MIME-006-01), Fondation Pour la Recherche Médicale (grant DMI20091117233), and European Union FP7 project ANTIGONE (grant 278976).

Potential conflict of interest. Both authors: No reported conflicts.

Both authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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