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ORIGINAL INVESTIGATION

Andreas Lucht · Roland Grunow · Christian Otterbein
Peggy Möller · Heinz Feldmann · Stephan Becker

Production of monoclonal antibodies and development of an antigen capture ELISA directed against the envelope glycoprotein GP of Ebola virus

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Abstract Ebola virus (EBOV) causes severe outbreaks of Ebola hemorrhagic fever in endemic regions of Africa and is considered to be of impact for other parts of the world as an imported viral disease. To develop a new diagnostic test, monoclonal antibodies to EBOV were produced from mice immunized with inactivated EBOV species Zaire. Antibodies directed against the viral glycoprotein GP were characterized by ELISA, Western blot and immunofluorescence analyses. An antigen capture ELISA was established, which is specific for EBOV-Zaire and shows a sensitivity of approximately 10^3 plaque-forming units/ml. Since the ELISA is able to detect even SDS-inactivated EBOV in spiked human sera, it could complement the existing diagnostic tools in the field and in routine laboratories where high containment facilities are not available.

Keywords Ebola virus · Ebola hemorrhagic fever · Monoclonal antibodies · Antigen capture ELISA

A. Lucht (✉) · R. Grunow · C. Otterbein
Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, 80937 Munich, Germany
E-mail: andreaslu@gmx.de
Tel.: +49-162-6014031
Fax: +49-89-31683292

P. Möller · H. Feldmann · S. Becker
Institute of Virology, Philipps-University Marburg, Robert-Koch-Strasse 17, 35037 Marburg, Germany

Present address: C. Otterbein
Authority of the district of Rosenheim, Public Veterinary Office, Am Klafferer 3, 83043 Bad Aibling, Germany

Present address: H. Feldmann
Special Pathogens Program, National Microbiology Laboratory, Health Canada, Canadian Science Centre for Human and Animal Health, Winnipeg, Canada

Introduction

Ebola virus (EBOV) was initially identified in 1976 after simultaneous outbreaks in Sudan and Zaire (Democratic Republic of Congo) [50]. In the following years more severe outbreaks of Ebola hemorrhagic fever (EHF) in Zaire, Sudan, Gabon, Republic of Congo and Uganda demonstrated its serious threat to humans living in endemic areas in central Africa. The introduction of EBOV-Reston into the United States in 1989, 1990 and 1996 [7, 8, 16] and to Siena/Italy in 1992 [51] by imported macaques from Luzon Island/Philippines showed that EBOV is not restricted to Africa. Moreover, a laboratory infection in Porton Down/England in 1976 [9] as well as the required treatment of EHF patients in Switzerland in 1994 [25] and in South Africa in 1996, causing the infection and death of a nurse [34], proved that there is also a certain risk of EBOV infections for humans in developed and non-endemic countries.

Four different species in the genus Ebola-like viruses were identified: EBOV-Zaire, EBOV-Sudan, EBOV-Ivory Coast, and EBOV-Reston. EBOV and Marburg virus (MBGV) [41] make up the family of Filoviridae, named after the characteristic filamentous form of the virions [21]. The Filoviridae belong to the order Mononegavirales [45].

The nucleocapsid complex of EBOV consists of the nonsegmented negative-strand RNA genome, the nucleoprotein (NP), the RNA-dependent RNA-polymerase L, the polymerase cofactor VP35, and the EBOV-specific transcription activator VP30. The structural proteins VP40 and VP24 represent viral matrix proteins connecting the nucleocapsid with the viral envelope. The only envelope glycoprotein of EBOV, GP, is an integral membrane protein, which forms the spike-like protrusions at the surface of the virions [10, 11]. In infected cells, GP is transported to the cell surface and is released into the culture medium not only via incorporation into mature viral particles but also as nonvirion

molecules [48]. GP is endoproteolytically processed during intracellular maturation into two subunits, which are connected by a disulfide bond: the peripheral subunit GP1 and the smaller GP2, which is membrane anchored [47]. The primary transcript of the GP gene is the non-structural small soluble GP (sGP), which is secreted by EBOV-infected cells [40, 46]. sGP is also found in high quantities in the serum of infected patients. The expression of the full-length GP needs a transcriptional editing [48].

Sensitive and rapid diagnostic tools are essential for detecting EBOV infections to implement public health measures at the early stages of an outbreak. Fast and reliable assays should not only be available for specialized biosafety laboratories but also for routine diagnostic laboratories. The main goal of this study was to produce monoclonal antibodies (mAbs) to EBOV GP and develop an antigen capture ELISA, which could be handled and distributed easily, and was also able to detect viral antigen in inactivated specimens.

Material and methods

Virus preparation

EBOV-Zaire strain Mayinga, EBOV-Sudan strain Boniface, EBOV-Ivory Coast strain Ivory Coast, EBOV-Reston strain Reston, and MBGV strain Musoke were grown in Vero cells and purified as described previously [31]. For immunization, purified EBOV-Zaire was inactivated by incubation with β-propiolactone (Sigma, Chemicals, St. Louis, MO; dilution 1:1,000) overnight. Thereafter, the samples were incubated for 1 h at 60°C in the presence of 1% Triton X-100. The concentration of viral structural proteins was approximately 1 mg/ml. To establish the capture ELISA, cell culture supernatants of EBOV-Zaire infected cells were used [approximately 10^6 plaque-forming units (pfu)/ml], inactivated by 1% SDS. For the screening of mAbs, purified and 100–500 times concentrated cell culture supernatant of EBOV-Zaire (inactivated by 1% SDS) was used.

Expression of recombinant antigen

Subconfluent HeLa cells (5×10^5 in 7-cm^2 wells) were infected with the recombinant vaccinia virus MVA-T7 at a multiplicity of infection of 3–5 pfu/cell [43]. MVA-T7 expresses the T7-RNA polymerase in the infected cells. At 1 h post infection (p.i.), cells were transfected with 1 µg of plasmids encoding EBOV GP using the lipofectin precipitation technique [12]. At 12 h after transfection, cells were fixed and subjected to immunofluorescence and Western blot analysis.

Development of mAbs

The hybridoma technique was used for the development of specific mAbs [14, 22]. Six-week-old female BALB/c mice (Charles River, Sulzfeld, Germany) were immunized intraperitoneally with inactivated antigen of EBOV-Zaire. One mouse was splenectomized at day 3 after the last boosting. The spleen cells were fused with the X63Ag8.653 myeloma cell line [20].

Cell culture supernatants of growing hybridoma cells were screened by ELISA for EBOV specificity and those hybridoma cell lines producing specific antibodies were recloned four to six times by limiting dilution to ensure monoclonality [28].

Isotype determination of immunoglobulins

Immunoglobulin isotypes were determined using the Isostrip mouse mAb isotyping kit (Boehringer Mannheim, Germany) and results were confirmed by ImmunoPure mAb isotyping kit (Pierce, Rockford, IL) according to manufacturer's instructions.

Western blotting

The specificities of the mAbs were evaluated by Western blot analysis with virion-associated proteins of EBOV-Zaire, EBOV-Ivory Coast, EBOV-Sudan, EBOV-Reston and MBGV. For identification of viral proteins recognized by the mAbs, recombinant EBOV structural proteins were used [2, 3]. HeLa cells were infected with the recombinant vaccinia virus MVA-T7 and subsequently transfected with plasmids containing the gene for the EBOV glycoprotein GP. At 12 h after transfection, the cells were lysed and lysates were separated by SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA). Membranes were cut into strips and incubated with the respective mAbs as described earlier [3]. SuperSignal Chemiluminescent Substrate (Pierce) was used for detection.

Immunofluorescence analysis

To investigate the reactivity of the mAbs in immunofluorescence analysis, HeLa cells, grown on glass cover slides, were infected with MVA-T7 and transfected with plasmids expressing the EBOV structural protein GP. Cells were fixed with 3% paraformaldehyde at 8 h p.i. and subjected to immunofluorescence analysis using the obtained mAbs and a rhodamine-conjugated donkey anti-mouse antibody as described previously [4]. As a control, mock-infected and vaccinia virus-infected cells without transfection were analyzed.

Large-scale production of specific mAbs

The GP-specific cell lines 1G12 and 3B11 were adapted to protein-free PFHM II medium supplemented with Glutamax 2 (Life Technologies, Paisley, Scotland). Cells were first grown in small tissue flasks (Nunc, Wiesbaden, Germany). 1G12 and 3B11 were then expanded in Celline flasks and the Tecnomouse production system (Integra Biosciences, Lowell, MA) in protein-free medium with high glucose (4.5 g/l) and high glutamine (4 mM) in the presence of 150 mg/l gentamycin (all Life Technologies).

Purification and labeling of mAbs

The mAbs 1G12 and 3B11 were purified on a protein A-Sepharose CL-4B column (Pharmacia, Freiburg, Germany) according to manufacturer's instructions and stored in PBS/0.02% Thiomersal (Sigma Chemicals).

Aliquots of both purified mAbs were conjugated to horseradish peroxidase (POD; Boehringer Mannheim, Germany) according to Wilson and Nakane [32, 49] and stabilized with 2% bovine serum albumin and 0.02% Thiomersal (provided by Seramun Diagnostica, Dolgenbrodt, Germany).

Antigen capture ELISA

Both mAbs were used to develop an antigen capture ELISA recognizing EBOV GP. Nunc-Immuno MaxiSorp 96-well plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 50 µl of different concentrations of mAbs 1G12 or 3B11 in carbonate buffer (pH 9.0). After washing five times with PBS/0.05%

Tween 20, plates were incubated for blocking with 100 µl PBS containing 5% or 10% of various supplements with or without 0.05% Tween 20 at 37°C for 60 min to prevent unspecific binding. The used supplements were: skim milk, fetal calf serum, bovine serum albumin, human serum albumin (all Sigma Chemicals), horse serum, goat serum or rabbit serum (all Life Technologies). Plates were washed, 50 µl EBOV antigen (diluted in PBS 1:1,000 or as indicated) were added and incubated for 1 h at 37°C. After another washing, 50 µl of different concentrations of POD-conjugated mAbs 3B11 or 1G12 (diluted in PBS/2% skim milk/0.05% Tween 20) were used as detecting antibody at 37°C for 60 min. After five washings, 50 µl TMB substrate solution were added for 15 min at room temperature. The reaction was stopped by 50 µl 0.25 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 620 nm using a DigiScan plate reader (Asys Hitech, Eugendorf, Austria) and MikroWIN 2000 software (Mikrotek, Overath, Germany). Cut-off levels were set by calculating the mean of ten negative controls plus three standard deviations.

Results

Production of mAbs

BALB/c mice were immunized with inactivated antigen of EBOV-Zaire. After the first prime, the mice were boosted three times. Three days after the last booster, the mouse with the highest antibody titer was splenectomized and the spleen cells were fused with a myeloma cell line.

After repeated cloning, two monoclonal hybridoma cell lines 1G12 and 3B11 producing specific mAbs to EBOV were selected, which were both of immunoglobulin isotype IgG1χ.

Characterization of mAbs

The obtained mAbs were investigated by Western blot analysis with a sensitive chemiluminescent detection system using virion-associated proteins and recombinant EBOV structural proteins. Transfected HeLa cell lysates or EBOV structural proteins were separated by SDS-PAGE and subsequently electroblotted onto PVDF membranes. Membranes were cut into strips and incubated with the respective mAbs. The Western blot analysis revealed that the two mAbs were directed against the viral surface protein GP (Fig. 1A, B; lanes 1 and 2). Both antibodies reacted with GP1 and sGP, which was co-purified during the preparation of the virions [48]. The slightly different migration patterns of the virion-associated (Fig. 1A, B; lane 1) and the recombinant GP1 (Fig. 1A, B; lane 2) are probably caused by a different glycosylation pattern in Vero and HeLa cells [3]. The distinct signal at 69 kDa in lane 2, where the recombinant GP was separated, represents the nonglycosylated GP, which was absent in the virion-associated structural proteins (lane 1) [3]. The protein bands appearing between 40 and 60 kDa in lane 2 probably represents degradation products or sGP produced by limited RNA-editing mediated by the T7 polymerase [46]. The strength of reactivity against GP differed between the two GP-specific antibodies. While

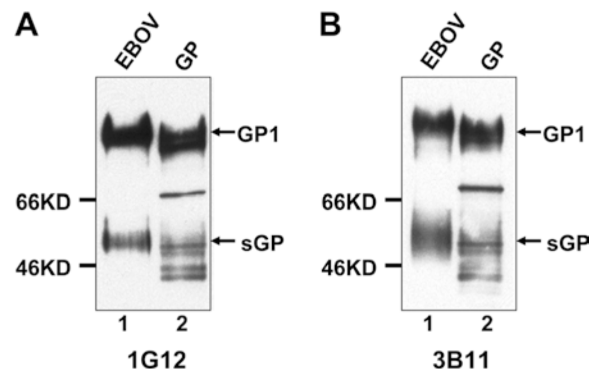

Fig. 1 Reactivities of mAbs in Western blot analysis using virion-associated EBOV-Zaire proteins and recombinant GP derived from EBOV-Zaire. Vero cells were infected with EBOV or HeLa cells with the recombinant vaccinia-virus MVA-T7 and transfected with plasmids containing the gene of GP. At 12 h p.i., cells were lysed; lysates were separated by SDS-PAGE and electroblotted onto PVDF membranes. Membranes were incubated with the respective mAbs. *Lane 1*: EBOV virion-associated proteins; *lane 2*: EBOV recombinant antigens. **A** mAb 1G12; **B** mAb 3B11 (*mAb* monoclonal antibody, *EBOV* Ebola virus, *GP* glycoprotein, *p.i.* post infection)

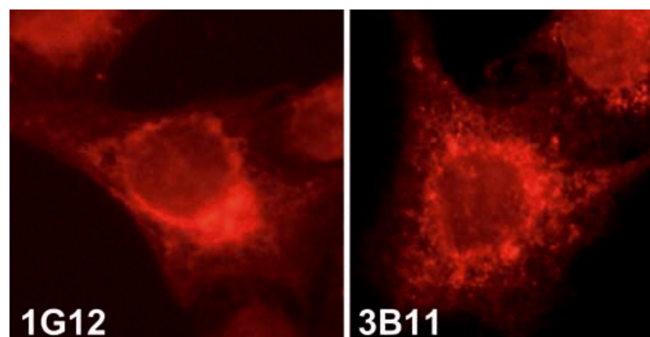

Fig. 2 Immunofluorescence analysis of mAbs. HeLa cells were infected with MVA-T7 and transfected with plasmids encoding EBOV structural protein GP. Infected cells were fixed with 3% paraformaldehyde at 8 h p.i. Cells were incubated with mAbs directed against the respective proteins and with a rhodamine-conjugated donkey anti-mouse antibody. **A** mAb 1G12 (50 µg/ml and exposed for 5 min to the X-ray film); **B** mAb 3B11 (12 µg/ml and exposed for 1 min to the X-ray film)

mAb 1G12 was used undiluted (50 µg/ml) and exposed for 5 min to the X-ray film, the mAb 3B11 was diluted 1:4 (12 µg/ml) and exposed 1 min to give the same signal strength.

The reactivity of the mAbs in immunofluorescence analysis was investigated with HeLa cells, expressing different recombinant EBOV-specific proteins. Cells were fixed with 3% paraformaldehyde at 8 hours p.i., and subjected to immunofluorescence analysis using the obtained mAbs. mAbs 1G12 and 3B11 recognized only cells that were transfected with plasmids encoding the GP-gene of EBOV. The perinuclear and filamentous structures probably correspond to the endoplasmic reticulum and Golgi apparatus (Fig. 2A, B). This localization is expected for the distribution of GP [29].

The immunofluorescence analyses of mock-infected cells and wild-type vaccinia virus-infected cells were completely negative as were cells that expressed other EBOV proteins (not shown).

Cross-reactivity of the obtained antibodies with other EBOV species or with MBGV, the other member of the family Filoviridae, was then analyzed. Structural proteins of EBOV-Sudan, EBOV-Ivory Coast, EBOV-Reston, and MBGV were isolated from the supernatant of infected cells, separated by SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were cut into strips, which were subsequently incubated with the mAbs. The specificity of the mAbs was tested using ELISA as well. The GP-specific mAbs did not recognize EBOV-Sudan or EBOV-Reston; however, mAb 1G12 did react with EBOV-Ivory Coast. Neither mAb reacted with MBGV. The results of the ELISA tests confirmed the data obtained by Western blot analyses (not shown).

Development of an antigen capture ELISA

To produce amounts of mAb sufficient to develop an antigen capture ELISA, the two hybridoma cell lines were adapted to protein-free medium. Using the Tecnomouse production system, both clones produced about 1 mg mAb per ml cell culture supernatant. Both mAbs 1G12 and 3B11 could be purified without any considerable loss of activity (data not shown). Of the purified mAbs, 15 mg 1G12 and 8 mg 3B11 were conjugated to POD. The reactivity of the mAbs towards EBOV was verified after the conjugation procedure.

The optimal concentrations of the mAbs for the antigen capture ELISA were determined in a cross-titration experiment. Best results were obtained with 5 μg/ml 3B11 as capture antibody and 20 μg/ml POD-conjugated 1G12 for detection. Further evaluation with different concentrations of viral antigen showed that using larger concentrations of 3B11 led to slightly higher absorbances but did not improve the detection limit. Higher concentrations of POD-conjugated 1G12 increased the background without improving the detection limit. Different blocking solutions were tested and the best results were found with 10% skim milk in PBS/0.05% Tween 20 (data not shown). The cut-off was set to an absorbance of 0.3, calculated by the mean of ten negative controls plus three standard deviations. GP was detected in the purified EBOV-Zaire antigen to approximately a dilution of 1:32,000. The detection limit for antigen derived from supernatants of infected cell cultures was 1:2,000, which corresponds to approximately 5×10^2 pfu/ml (Fig. 3A, B; open circles).

The ELISA was then tested on sera spiked with EBOV. Supernatant from EBOV-infected Vero cell culture was added at various dilutions to human sera. The cut-off for serum samples was set to an absorbance of 0.2. The assay was sensitive up to a final supernatant dilution of 1:1,000 (Fig. 4A; open circles). To check whether SDS inactivation impaired the sensitivity of the ELISA, 1% SDS was added to the spiked serum samples that were then tested. Although the measured absorbance had decreased, the sensitivity of the assay dropped only slightly (1:500; Fig. 4B; open circles). The sensitivity of the assay corresponded to approximately 10^3 pfu/ml serum.

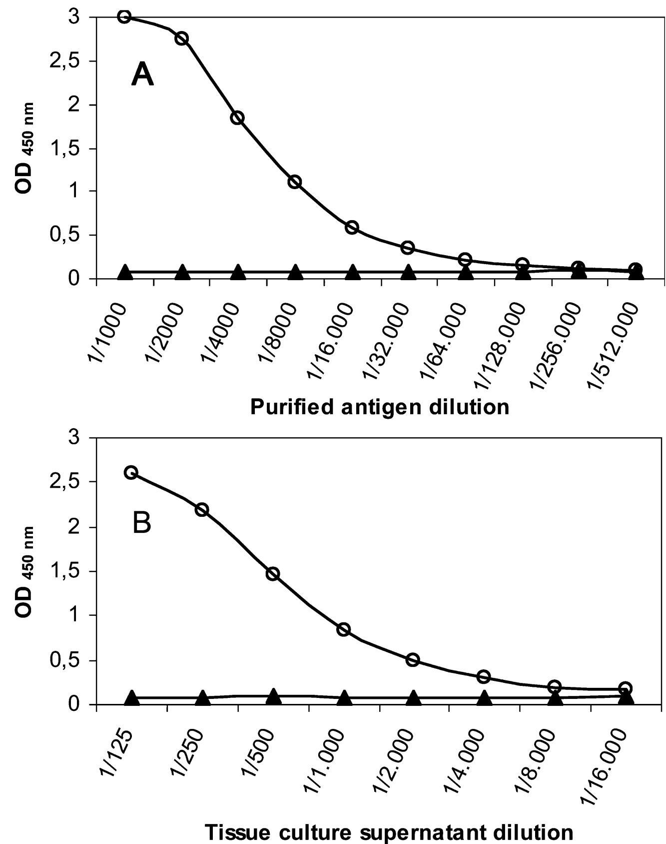

Fig. 3 ELISA results with purified antigen and tissue culture supernatant of infected Vero cells diluted in PBS. (Measured ODs >3 were shown as an OD of 3 in this figure.) **A** Purified antigen, **B** cell culture supernatant of infected Vero cells; *open circles* EBOV-Zaire, *filled triangles* MBGV (*MBGV* Marburg virus)

Discussion

Since the first outbreaks of EHF in Zaire and Sudan in 1976 diagnostic tools for EBOV have been developed and improved. Early diagnosis, however, still remains difficult. Serological tests all face the same problem that laboratory diagnosis of an acute infection is impossible because antibody response against EBOV is rather weak and often appears late in the course of disease [1, 37]. Nevertheless, several methods of antibody detection have been developed, which are easy to perform in field laboratories in Africa and which have been used for epidemiological studies [15, 17, 19, 42]. However, the results obtained were not very reliable, possibly because of cross-reactivities with other viruses [44].

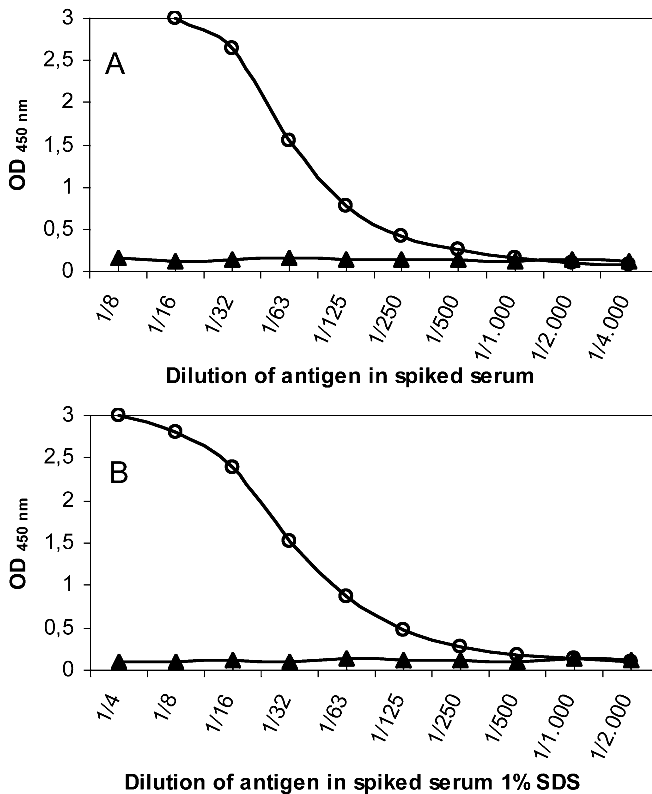

Fig. 4 Detection of antigen in human serum spiked with EBOV-Zaire and MBGV. (Measured ODs > 3 were shown as an OD of 3 in this figure.) **A** Untreated serum, **B** 1% SDS-inactivated serum; *open circles* EBOV-Zaire, *filled triangles* MBGV

Because of the presence of high titers of infectious virus in blood and tissues, antigen detection plays the most important role in early detection of EBOV infection. Viral antigen is detectable 3–6 days after the onset of symptoms. It disappears between days 7 and 16 [37]. An the initial stages of disease, antigen titers are similar in fatalities and survivors. During the following days, antigen titers decrease in survivors and finally disappear with recovery, whereas in fatalities antigen load rises until death [1].

Of the methods used to detect EBOV antigen, the most definitive method, virus isolation in cell cultures (Vero cells) [35] or animals (guinea-pigs) [6], can only be performed under biosafety level 4 conditions and is time consuming. This method is not suitable for outbreak detection and management. Electron microscopy is a sensitive method [13], but it needs special equipment and a qualified and experienced investigator. Immunofluorescence stainings of blood and impression smears from organs are rapid and sensitive [36], but often difficult to interpret. Immunohistochemical methods are very sensitive and reliable especially for post-mortem diagnosis [52]. Although they are time consuming and not suitable for early diagnosis, these methods represent an appropriate tool for hemorrhagic fever surveillance in endemic areas.

At present, the most favorable methods for early diagnosis of EHF are reverse transcription (RT)-PCR and antigen detection ELISA. RT-PCR is a very fast and sensitive method for the detection of viral genomic RNA, which is able to detect virus from early acute disease throughout early recovery [27, 39]. The verification of positive results is required because of the risk of potential contaminations [26]. Fast and sensitive antigen-detecting ELISAs have been developed by several groups [5, 23, 24, 30, 33]. None of these tests, however, are commercially available.

Our aim was to develop mAbs against EBOV structural proteins that could be used for an antigen capture ELISA based on mAbs for both antigen capture and detection. The obtained GP-specific antibodies were of IgG1 subclass and reacted in Western blot and immunofluorescence analysis with both the virion-associated and the recombinant EBOV-Zaire GP. Whereas mAb 3B11 was specific for EBOV-Zaire, mAb 1G12 also reacted with EBOV-Ivory Coast. These results point to the fact that different epitopes are probably being recognized.

For the development of an antigen capture ELISA, GP was regarded to be the most promising target. The glycoprotein GP can be found on the surface of virions and infected cells, and is also released into cell culture supernatant and blood [48].

The antigen capture ELISA, developed by Ksiazek and coworkers [23, 24], uses a pool of five mouse mAbs for coating; the specificity of these mAbs has not been published. Another antigen capture ELISA, developed by Niikura et al. [33], uses NP-specific mAbs. Both published ELISAs use a polyclonal rabbit serum for detection. Polyclonal antisera often show higher sensitivity and avidity than mAbs. On the other hand, mAbs may have the advantages of higher specificity and their availability in unlimited amounts of the same quality.

Our antigen capture ELISA was developed using specific mAbs only: one for capture and another one for detection of EBOV antigen. First investigations showed that the mAb 3B11 could be used as capture antibody and the POD-conjugated mAb 1G12 as detection antibody in an ELISA for specific detection of the EBOV-Zaire surface protein. The purified EBOV-Zaire antigen could be detected up to a dilution of approximately 1:32,000, the supernatant of infected cell culture could be detected up to 1:2,000, which corresponds to approximately 5×10^2 pfu/ml. During the purification procedure of viral antigen, the amount of virions is approximately 100–500 times concentrated compared to cell culture supernatant. Thus, one would expect that signal strength of the two preparations reflected this ratio and the concentrated virus preparation should display a 100–500 times higher titer. The ratio of the detection limits (cell culture supernatant/purified antigen), however is only 1:16. The difference to the expected value could be explained by the high amount of soluble GP, which is released into the medium of EBOV-infected cells [48]. Although a small amount of sGP is copurified with the virions (Fig. 1), the vast majority of the soluble GP is not purified together with the virions.

These findings are interesting since sGP is also found in high amounts in the serum of EBOV-infected patients.

EBOV antigen could be detected in spiked human sera. The ELISA showed a sensitivity of approximately 10^3 pfu/ml serum. The inactivation with 1% SDS did not lead to a substantial loss of sensitivity. Given that a patient suffering from EHF has serum titers of approximately 10^6–10^8 viral particles per ml plus the huge amount of sGP, it is reasonable to expect that the sensitivity of the ELISAs is sufficiently high to detect even early stages of infection.

This ELISA is able to detect specifically EBOV-Zaire, the virus which caused severe outbreaks of EHF in Zaire, Gabun, the Republic of Congo and Uganda in the last decades. EBOV-Zaire is regarded to be the most dangerous EBOV species with a fatality rate of more than 80% [38]. Therefore, this ELISA can be a very helpful tool to test people at risk and possibly infected persons, or to confirm suspected cases during an ongoing outbreak caused by EBOV-Zaire. Nevertheless, the usability of this ELISA is limited during outbreaks of unknown hemorrhagic fever as it does not detect EBOV-Sudan. In such a setting it could be effectively applied in combination with the previously published VP40-ELISA detecting all known EBOV species [28].

In conclusion, the obtained mAbs to EBOV GP were suitable for developing a specific and sensitive antigen detection system. The established ELISA needs to be evaluated with sera from EHF patients and other clinical specimens. This test might then represent a valuable tool for rapid laboratory diagnosis of EHF outbreaks in Africa and of imported cases in other parts of the world.

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