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Evidence for occurrence of filovirus antibodies in humans and imported monkeys: do subclinical filovirus infections occur worldwide?

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Abstract. In the present serologocal study 120 monkey sera from different species originating from the Philippines, China, Uganda and undetermined sources and several groups of human sera comprising a total of 1288 specimens from people living in Germany were examined for the presence of antibodies directed against filoviruses (Marburg virus, strain Musoke/Ebola virus, subtype Zaire, strain Mayinga/Reston virus). Sera were screened using a filovirus-specific enzyme-linked immunosorbent assay (ELISA). ELISA-positive sera were then confirmed by the indirect immunofluorescence technique, Western blot technique, and a blocking assay, and declared positive when at least one cornfirmation test was reactive. Altogether 43.3% of the monkey sera and 6.9% of the human sera reacted positively with at least one of the three different filovirus antigens. The blocking assays show that antibodies, detected in the sera, are directed to specific filovirus antigens and not caused by antigenic cross-reactivity with hitherto unknown agents. Data presented in this report suggest that subclinical filovirus infections may also occur in humans and in subhuman primates. They further suggest that filoviruses are not restricted to the African continent.

Introduction

The prototypes of the family Filoviridae [22] are Marburg virus (MBG) and Ebola virus (EBO) which both cause severe hemorrhagic disease. Filovirus infections were unknown until 1967, when 25 persons in Germany (cities of Marburg and Frankfurt) and Yugoslavia (city of Belgrad) became infected with MBG after contact with tissues and blood from African green monkeys (*Cercopithecus aethiops*) imported from Uganda. Seven of these cases resulted in death. In addition, six secondary non-lethal cases of infection occurred [26, 30]. All African

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green monkey infected experimentally with the virus died [29, 35]. Suprisingly, no specific antibodies were found in sera from monkeys captured in the same area where the shipment to Germany and Yugoslavia had originated. Since then, sporadic, virologically confirmed Marburg disease cases have occurred in various parts of Africa: South Africa, 1975 [12], Kenya, 1980 [39], and Kenya, 1987 [24]. The geographically and temporally distinct MBG outbreaks appear to have been due to genetically distinguishable, but antigenetically closely related virus strains [24].

EBO first emerged in two major outbreaks which occurred almost simultaneously in Zaire and Sudan in 1976 [41, 42]. There were more than 550 cases and more than 430 resulted in death [2, 18]. Two subtypes were isolated which differ in pathogenicity, antigenicity, and genomic composition [3, 9]. They were morphologically identical with, but serologically distinct from MBG. The case fatality rate of the Zaire and Sudan subtypes was 88% and 53%, respectively. However, secondary and tertiary cases had lower fatality rates, suggesting attenuation of the virulence with human-to-human pasasages. In 1977, another fatal case was reported from Zaire [13], and in 1979, EBO hemorrhagic disease occurred again in Sudan with 34 cases of which 22 resulted in death [1, 43]. Serological studies suggest that infections with EBO or related viruses have occurred in several African countries [15]. No antibodies have ever been found in specimens of feral animals, including more than 200 monkeys collected in Zaire and Sudan.

In 1989, a third filovirus, serologically related to EBO, was isolated when an explosive epizootic occurred in monkeys (*Maccaca fascicularis*) imported to the United States from the Philippines [16]; most of the monkeys died. The agent, called Reston virus (RES), is infectious to humans but does not seem to cause serious human disease [5, 15].

Filoviruses are enveloped, nonsegmented negative-stranded RNA viruses. The genome has a molecular weight of approximately 4.2×10^6 [31]. Virions contain at least seven structural proteins, nonstructural proteins have not yet been detected. The structural proteins are: an RNA-dependent RNA polymerase (L protein), a glycoprotein (GP), a major (NP) and a minor (VP30) nucleoprotein, two proteins (VP40, VP24) associated with the viral membrane, and VP35 which is thought to be the P (NS) protein equivalent of paramyxo- and rhabdoviruses [10, 11, 24, 27, 33, 34].

The natural reservoirs for human infection with MBG and EBO and the natural reservoir of RES are unknown. Serological studies employing enzyme-linked immunosorbent assay (ELISA) or immunofluorescence assay have suggested that EBO antibodies are present in a number of human and animal populations in parts of Africa [17, 37, 40]. Recent serological investigations using indirect immunofluorescence assay (IFA) and Western blot were performed in connection with the RES epizootic in the Unites States on sera of persons with varying levels of exposure to monkeys. Of these sera 7.6% reacted positively with one or more of the different filovirus antigens. In addition, randomely selected sera of persons without known exposure were tested by the same assays resulting in 2.7% positives [6–8].

In this study we analyzed sera of monkeys from different species and countries as well as human sera from different parts of Germany. All sera were examined using IFA, ELISA, and Western blot techniques. Sera positive to one of the filovirus antigens were further analyzed in a blocking assay.

Materials and methods

Viruses and cell line

The Musoke strain of MBG isolated 1980 in Kenya [39], the Mayinga strain of EBO, a Zaire subtype isolated in 1976 [42], and the RES [6, 16] were used. The viruses were grown in E6 cells, a cloned cell line of Vero cells (American Type Culture Collection CRL 1586).

Growth and purification of viruses

E6 cells were infected with the different viruses at a multiplicity of infection of 10^{-2} plaque-forming units/cell. Following an adsorption period of 30 min at 37°C, infected cells were incubated in Dulbecco medium containing 2% fetal calf serum. Virus was harvested 8 days post infection (cytopathic effect approximately 85%) for MBG and EBO. RES was harvested 14 days post infection, and the medium was changed at day 7 post infection. Virus purification was performed as described previously [10, 23, 27, 32].

Serum samples

Monkey sera were obtained from healthy animals imported to Germany and Switzerland from the Philippines (*Macaca fascicularis*, imported in November 1989 and January 1990; 31 sera), China (*Macaca mulatta*, imported in September 1990; 37 sera), Uganda (*Cercopithecus aethiops*; colonies founded in 1978; 43 sera) and species from undetermined origin and date of import (*Macaca nemestrina*, 2 sera; *Macaca mulatta*, 7 sera). Human sera were obtained from following sources: 673 sera sent for routine diagnostics to the department of Virology at the University of Marburg, 475 sera of persons undergoing routine health control, 89 sera from blood donors, 56 sera from persons (taken in 1972) with contact to patients suffering of Marburg hemorrhagic disease, and 32 sera from persons, living in the surrounding of Greifswald (former GDR).

Western blot analysis

Viral proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide slabgels (SDS-PAGE) as described by Laemmli [25]. Preparative gels were loaded with 20 µg viral proteins and were run at 30 mA for 1.5 h. After electrophoresis proteins were blotted onto PVDF-membranes (Millipore, #P-15552) by semi-dry blot technique. The PVDF-blots were cut into stripes which then were incubated with human and monkey sera at a dilution of 1:100 in phosphate-buffered saline (PBS)/0.2% Tween 20. Horseradish peroxidase (HRP)-coupled rabbit anti-human IgG was used at a dilution of 1:300 in PBS/0.2% Tween 20 for detecting bound antibodies.

Enzyme-linked immunosorbent assay

96-well polystyrene plates (Flacon, #3072) were coated overnight with suspensions of SDS-inactivated virus at a concentration of 1 µg/ml. The coating buffer contained 2.93 g sodium hydrogen carbonate and 1.59 g dihydrogen sodium carbonate/l H_2O, pH 9.8. Subsequently, plates were washed three times with PBS/0.2% Tween 20 and nonspecific binding sites were blocked by incubation in PBS containing 3% bovine serum albumin for 16 h at 4°C. Plates were washed again (see above) and incubated with human and monkey sera ta a dilution of 1:100 in PBS/0.2% Tween 20 for 1 h. After further washing bound antibody was detected with HRP-labelled anti-human-IgG antibodies at a dilution of 1:500 in PBS/0.2% Tween 20. The absorbance was measured at 492 nm with 650 nm reference wavelength.

Blocking assay

Human and monkey sera positive to antigens from one of the three different filoviruses (MBG, EBO, RES) were incubated in 96-well polystyrone plates coated with either the homologous (e.g., MBG-positive sera with MBG antigen) or heterologous antigen preparation (MBG-positive sera with RES antigen, EBO-positive sera with MBG antigen and RES-positive sera with EBO antigen) for 16 h at 4°C. Subsequently, the preincubated sera were removed and examined on ELISA plates coated with the homologous antigen (e.g., MBG-positive sera with MBG antigen) or in Western blot analysis as described above.

Indirect immunofluorescence

Monolayers of virus-infected cells (3 days post infection) were dispersed with trypsin, washed with PBS, and applicated to uncoated areas of epoxy-coated slides (Bio Merieux). Infected cells were incubated for further 24 h at 37°C, washed with PBS, fixed with cold (−20°C) acetone for 20 min, and air-dried (can be stored at −20°C for several months). For antibody screeening slides were incubated with human and monkey sera at dilutions of 1:40 and 1:10, respectively, in a buffer containing 145 mM NaCl, 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 7 mM sodium barbital; 10 mM sodium azide, pH 7.2. Detection was performed using FITC-conjugated anti-human IgG antibodies (DAKO).

Results

Monkey sera

A total of 120 monkey sera from different species originating from different countries were tested for the presence of antibodies directed against filoviruses by ELISA, IFA, and Western blot techniques. None of the monkeys was known to have suffered from MBG or EBO hemorrhagic fever or similar diseases. The results are summarized in Table 1. All the sera were first screened in an ELISA, the positive ones were further examined either by IFA or Western blot technique. Sera were declared as positive when the results of at least two assays corresponded. Altogether 43.3% of the sera reacted positively with one of the used filoviruses (MBG, EBO, RES).

Table 1. Filovirus-specific antibodies in monkey sera[a]

Origin	Species	Date of import	Total number of sera/sera with filovirus antibodies
Philippines	*Macaca fascicularis*	11/1989 1/1990	31/12 (39%)
Uganda	*Cercopithecus aethiops*	1978	43/32 (76%)
?	*Macaca nemestrina*	?	2/– (0%)
?	*Macaca mulatta*	?	7/2 (28%)
China	*Macaca mulatta*	1990	37/6 (16%)

[a] 120 monkey sera from different origins were screened for antibody prevalence against filoviruses by ELISA and confirmed using indirect immunofluorescence and Western blot techniques

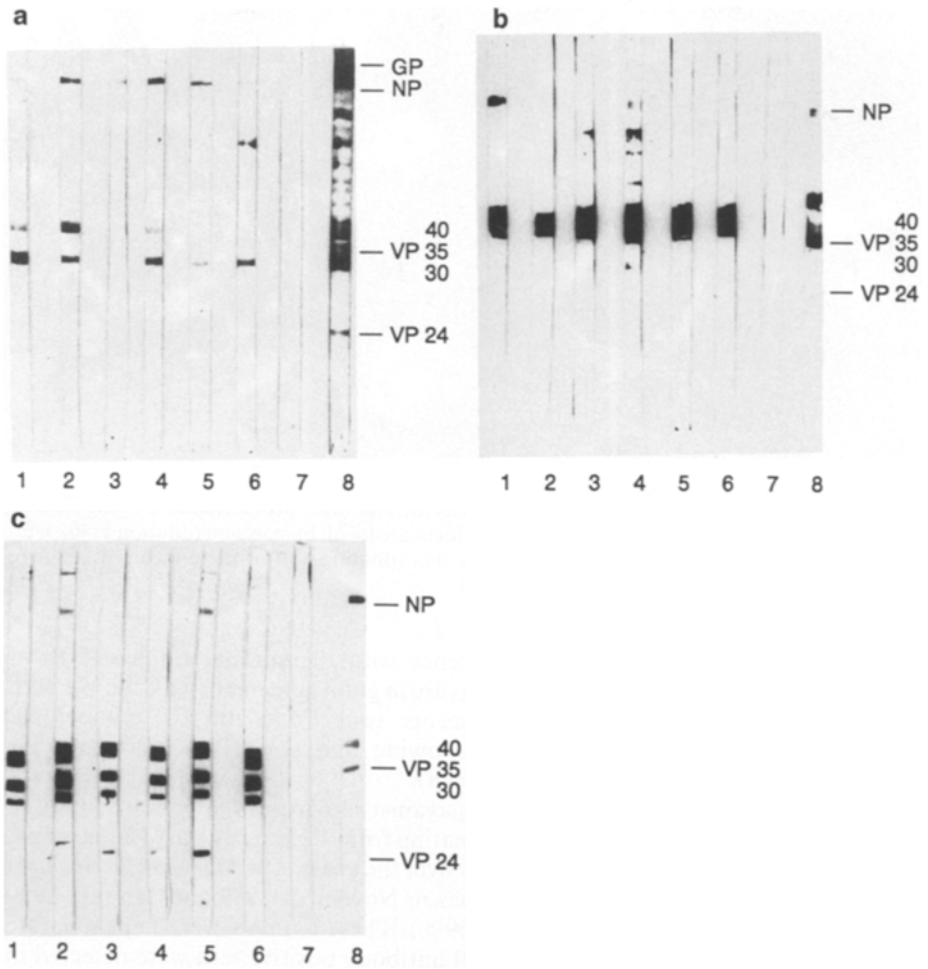

Fig. 1a–c. Immunoblot with monkey sera. Viral antigens (Ebola, Marburg, Reston virus; EBO, MBG, RES, respectively) were purified by gradient centrifugation and inactivated with SDS at a final concentration of 1%. Virion proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. The incubation was performed with sera at a dilution of 1:100 for 1 h and immunocomplexes were detected with horseradish peroxidase (HRP) rabbit anti-human-IgG. **a** EBO-proteins; **b** MBG-proteins; **c** RES-proteins. *1–6:* Positive monkey sera; *7:* negative monkey serum; *8:* positive control serum: **a** Anti-EBO human convalescent serum; **b** anti-MBG human convalescent serum; **C** human serum reacting specific with RES antigen. The numbers in **a, b,** and **c** do not represent identical sera

Western blot results were declared positive when two or more of the known virion proteins were detected. Most of the positive sera reacted with several viral proteins (NP, VP40, VP35, VP30), only a few reacted with VP24. Hoever, none of the investigated sera reacted with the GP and the L protein of any of the three filoviruses (Fig. 1). Some sera reacted positively with more than one filovirus.

In IFA analyses, positive sera showed the typical staining seen during filovirus infections in infected cells (Fig. 2c). Intracytoplasmic viral inclusion bodies consisting of viral nucleocapsid were predominantly visuable [28, 29]. None of the

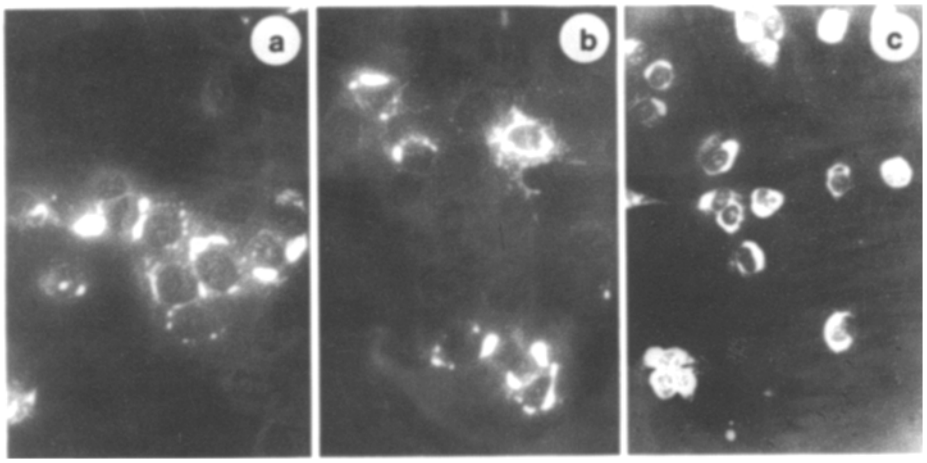

Fig. 2a–c. Indirect immunofluorescence. **a** E6 cells infected with MBG, incubated with human sera (dilution 1:40); **b** E6 cells infected with RES incubated with human sera (dilution 1:40); **c** E6 cells infected with RES incubated with monkey sera (dilution 1:10). Immunocomplexes were detected with FITC-conjugated anti-human IgG

sera showed a positive immunofluorescence with the surface antigen (GP) of filovirus-infected cells. These observations are in good agreement with the Western blot results. Additional immunofluorescence tests using anti-GP monoclonal antibodies, however, resulted positive showing that the GP is present in the membrane of infected cells (data not shown).

The highest prevalence of antibodies against filoviruses (76%) were found in the group of *Cercopithecus aethiops* originating from Uganda at least 12 years ago, which have been kept together in colonies over the years. The *Macacae fasciculares* which were imported from the Philippines in November 1989 and January 1990 also reacted positive to a high degree (39%). These animals were kept separate during quarantine. The lowest number of antibody positive sera were detected in the group of *Macacae mulattae* imported from China in September 1990 (16%). The two *Macacae nemestrinae* from an unknown source were negative, but the number of monkeys examined is too low to make any statement about this species (Table 1).

Human sera

A total of 1288 human sera of different origins were analyzed for antibodies directed against different filovirus antigens (MBG, EBO, RES). Sera were screened using an ELISA. ELISA-positive sera were further examined by more reliable techniques (IFA and Western blot) to verify the results. None of the tested people had a record of former MBG or EBO hemorrhagic fever or any comparable disease. A group of 56 sera were taken in 1972 from persons, who had been at risk by contact to patients suffering from MBG hemorrhagic disease. The various groups of human sera from different origin showed no statisticly significant differences concerning antibody prevalence against filoviruses, this applies also to the 56 sera of the risk group. They were, therefore, further treated as one group.

Table 2. Filovirus-specific antibodies in human sera[a]

	Ebola (Zaire)	Marburg (Musoke)	Reston
Positive sera found by ELISA[b]	11 (0.85%)	34 (2.6%)	44 (3.4%)
Confirmed by immunofluorescence[c]	75%	85%	80%
Confirmed by immunoblot[d]	77%	80%	66%

[a] 1288 Human sera were tested by antibody prevalence against filoviruses. The screening test was done by ELISA. The following confirmatory tests by immunofluorescense and immunoblot
[b] Serum dilution 1:100
[c] Serum dilution 1:40
[d] Serum dilution 1:100

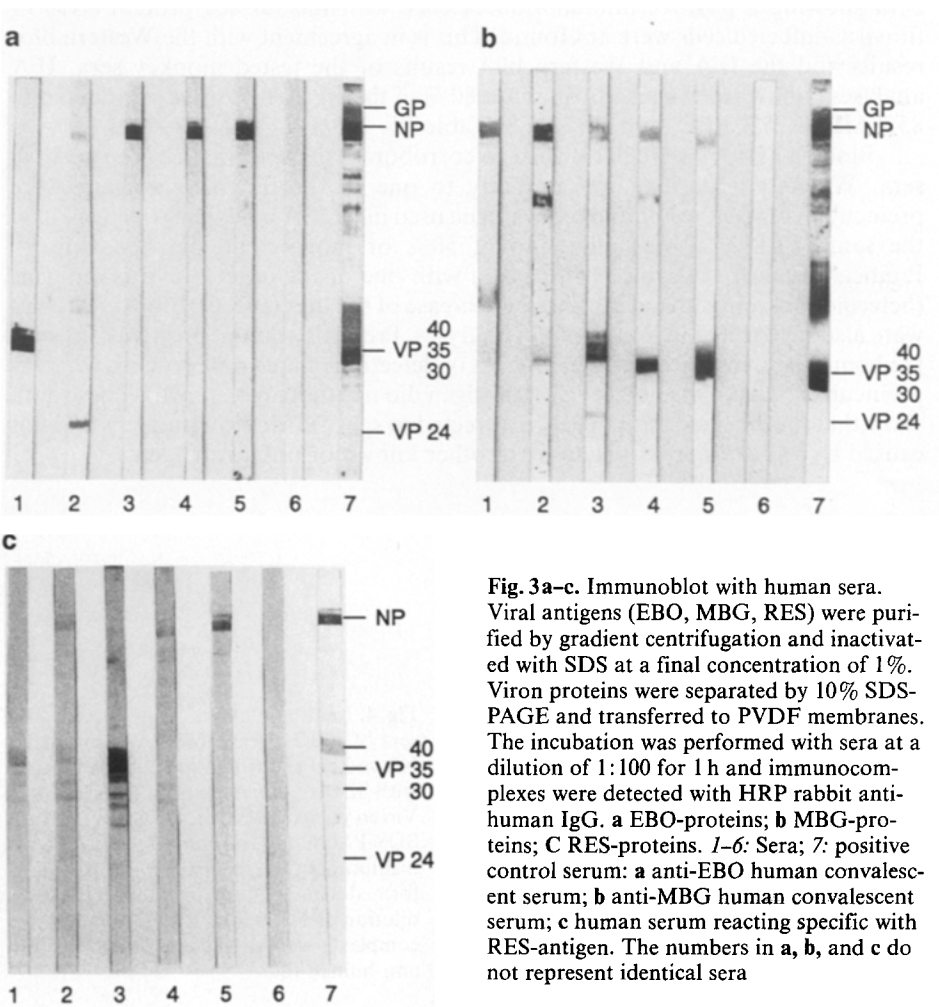

Fig. 3a–c. Immunoblot with human sera. Viral antigens (EBO, MBG, RES) were purified by gradient centrifugation and inactivated with SDS at a final concentration of 1%. Viron proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. The incubation was performed with sera at a dilution of 1:100 for 1 h and immunocomplexes were detected with HRP rabbit anti-human IgG. **a** EBO-proteins; **b** MBG-proteins; **C** RES-proteins. *1–6:* Sera; *7:* positive control serum: **a** anti-EBO human convalescent serum; **b** anti-MBG human convalescent serum; **c** human serum reacting specific with RES-antigen. The numbers in **a, b,** and **c** do not represent identical sera

Table 2 shows the results of the seroepidemiological study with 1288 sera. Altogether 89 sera (6.9%) were ELISA positive to filoviruses with 2.6% for MBG, 0.85% for EBO, and 3.4% for RES. Out of the 89 filovirus-positive sera 34 (38.2%) reacted with MBG, 11 (12.4%) with EBO, and 44 sera (49.4%) with RES.

In western blot analyses sera were considered positive when they would stain with at least two of the viral proteins. In accordance with the monkey sera none of the positive sera reacted with the GP and L protein of the filovirus antigens used in this assay (Fig. 3). Most of the sera reacted with the NP, less with one or more of VP40, VP35, and VP30, only a few reacted with VP24. Antibodies directed against the L protein could not be found even in sera of persons with known history of MBG or EBO hemorrhagic disease (Fig. 4). Western blot analyses confirmed an average of 74% of the ELISA-positive sera, in detail 80% MBG, 77% EBO, and 66% RES (Table 2).

IFA-positive sera showed an intracytoplasmic immunofluorescence and no staining of the cell nucleus. Viral antigen in coarse and fine aggregates could be seen (Fig. 2). These aggregates consists of the viral nucleocapsid (inclusion bodies). Sera showing a positive immunofluorescence with the surface protein (GP) of filovirus-infected cells were not found. This is in agreement with the Western blot results and the IFA and Western blot results of the tested monkey sera. IFA analyses confirmed the results of about 80% of the ELISA-positive sera, in detail 85% MBG, 75% EBO, and 80% RES (Table 2).

Blocking assays were performed to corroborate the specificity of the positive sera. When sera positive for antibody to one of the filovirus antigens were preincubated with the homologous antigen used in ELISA, and then tested again in the same ELISA a titer reduction of 50% or more could be seen (Fig. 5). Preincubation of the same positive sera with one of the other filovirus antigens (heterologous antigen) did not show a decrease of the titer (Fig. 5). Blocking assays were also performed in Western blot analyses. Preincubation of positive sera with the homologous antigen resulted in a loss of detection of the viral proteins, whereas preincubation with the heterologous antigen did not (data not shown). These data show that the detected antibodies are directed to specific filovirus antigens and not caused by antigenic cross-reactivity to other known or unknown agents.

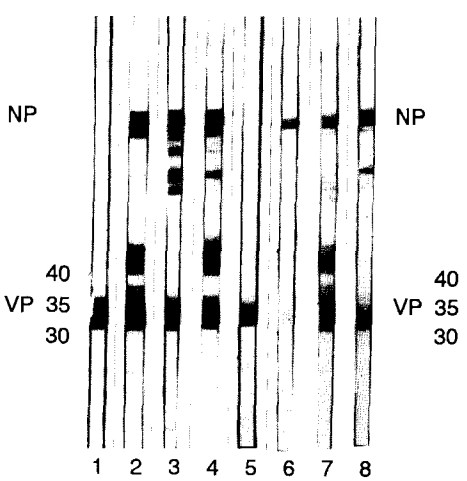

Fig. 4. Immunoblot with convalescent sera of MBG disease. MBG was purified by gradient centrifugation and inactivated with SDS at a final concentration of 1%. Virion proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. The incubation was performed with MBG-convalescent sera at a dilution of 1:100 for 1 h and immunocomplexes were detected with HRP rabbit anti-human IgG

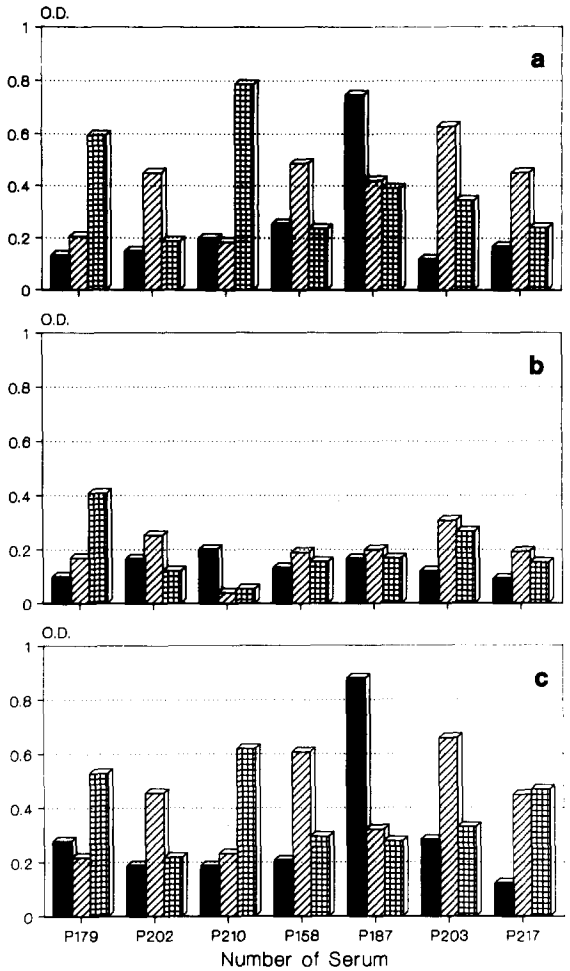

Fig. 5a–c. Blocking assay. Sera were preincubated with one of the three filoviruses and thereafter examined by enzyme-linked immunosorbent assay (ELISA) either with the same virus used in the previous step (homologous) or with another filovirus (heterologous). The absorbance given by the HRP-anti-human IgG was measured with an ELISA-reader (592 nm). **a** Without preincubation; **b** homologous preincubation; **c** heterologous preincubation; ■ Ebola-virus, ▨ Marburg-Virus, ▦ Reston-Virus

Discussion

Up to the end of 1989, filoviruses were believed to originate from the African continent exclusively. There are several reports, based on IFA assays, on the occurrence in various African populations of a considerable percentage of human sera positive for filovirus antibodies [14, 17, 19, 37, 40]. Similar findings in human sera from other continents are not known. Monkeys, on the other hand, especially those of the species *Cercopithecus aethiops*, had been identified as the source of infection in the case of the MBG outbreak from 1967 but regarding the high pathogenicity of the virus they were not believed to constitute the animal reservoir [35, 38]. Earlier findings of MBG-positive monkey sera of African and Asian origin [20, 21] could not be confirmed [36].

This situation has changed since October 1989, when a new filovirus (RES), antigenically related to EBO, was found in specimens of monkeys of the species *Macaca fascicularis* of Javanese origin, which suffered from hemorrhagic fever after their importation to the USA [4, 16]. Since this time it has become evident,

that filovirus infections – probably with a lower pathogenic potential [4] – may also be present in wild caught subhuman primates from Asia.

In the present report 120 sera from various monkey species of African, Asian and unknown origin were tested for the presence of filovirus antibodies, and a considerable proportion (43.3%) showed reactivity not only with RES antigen but also with EBO and MBG antigens. The reactions of monkey sera to filovirus antigens are characterized by the absence of antibodies against the GP and L protein. Reactions to the NP are frequent and especially VP40, VP35 and VP30 are heavily stained in Western blot analyses. Absence of reactivity to the GP may be due to the high degree of glycosylation of this protein and might be also attributed to the fact that the virus strains used for antigen preparation were certainly not identical with the viruses which had caused the antibody production in these animals. Similar patterns of reactivity are also encountered in some of the human MBG convalescent sera (Fig. 4). It has to be pointed out that serum titers to filoviruses in monkey sera are relatively low as compared to human convalescent sera. In earlier studies (Slenczka, unpublished) on monkey sera of African origin, this observation had led to the conclusion that these sera were not considered to be seropositive. However, the lower titers of monkey sera might again reflect the situation of antigenic differences between the strains used for antigen preparation and the virus strains causing the antibody production. In addition the low titers might also reflect antigenic differences between IgG of monkey and of human origin in regard to the specificity of the second antibody (rabbit anti-human IgG) used in ELISA and Western blot analyses. The occurrence of antibodies directed against filoviruses in *Macacae mulattae* imported from China would support the findings of RES-infected monkeys imported to the United States from the Philippines [16] and suggest that filoviruses are spread more widely over the world.

The suspicion that infections with filoviruses or with antigenically related viruses may be common in subhuman primates will have to be proved by efforts for virus detection using the polymerase chain reaction or virus isolations and by studies on the route of virus transmission in these animals.

Our findings of serological reactions to filovirus antigens or related viral antigens in human sera from central Europe raises several questions. Such reactivities have not been recognized in earlier studies, in which sera from the normal population were included as negative controls. In previous studies with MBG convalescent sera from 1967 we have regularly observed cross-reactivities with EBO antigens; however, sera from 120 human controls did not react with either antigen (Slenczka, unpublished). In these experiments SDS was not included in the antigen preparation. The denaturation of the antigens by SDS may uncover different (originally covered) epitopes which are unaccessible in the native protein and lead to positive reactions. On the other hand, a considerable proportion of the recently identified filovirus sera have also shown a positive reactivity in IFA where acetone-fixed preparations were stained and no SDS was included.

Cloning and sequencing data of filovirus genomes have revealed an evolutionary relationship of filoviruses to other nonsegmented negative-stranded RNA viruses among which they seem to be more closely related to paramyxoviruses than to rhabdoviruses [27, 34]. However, an antigenic relationship with any of the known paramyxoviruses and rhabdoviruses has never been documented for filoviruses.

Similar to the monkey sera, none of the human sera reacts with the GP and L protein of filoviruses. The number of L protein molecules per viron particle is very

low and was calculated to 46.6 for EBO [10] and 56 for MBG [24]. In addition it seems to be low immunogenic, which would also explain the negative reaction of the sera to the L protein. Absence of reactivity to the GP is probably due to its high degree of glycosylation or the fact that the virus antigens are not identical with the virus strains causing the antibody production as already discussed for the monkey sera. The fact that the antibody titers of the human sera are relatively low as compared to human convalescent sera might be due to the same circumstances. Most of the sera detected the NP and the VP40, VP35, and VP30 complex, whereas only a few reacted with the VP24. The fact that the majority of antibody-positive human sera stains two or more of the virion proteins is hardly compatible with a B cell activation of other origin which could lead to false-positive results.

The blocking assays with either homologous or heterologous antigens were performed to prove the specificity of antigen detection. The titer reduction after incubation with the homologous antigen and the non-reduction after incubation with the heterologous antigen suggest that the reaction is specific for the respective filovirus antigen, and that it is not directed against material of cells, which were used for virus propagation. In addition, the success of homologous immunoabsorption proves that the observed antibody titers cannot be attributed to a hypothetical cross-reactivity with known or unknown agents. If the reactions were caused by antigenic cross-reactivity or group-specific reactions one would rather expect a titer reduction with the homologous as well as the heterologous antigen.

As in the case of antibody findings in the monkey sera our results on human sera will have to be confirmed by further studies. The expression of virion proteins in procaryotic as well as eucaryotic systems is currently under way. These proteins can be used to develop more reliable screening tests, especially for field studies. The data presented in this report suggest that hitherto unknown filoviruses with variable pathogenic potential may be present in many parts of the world, and subclinical infections may occur frequently. Studies involving methods for virus detection with monoclonal antibodies or the polymerase chain reaction will be necessary to uncover the epidemiology and pathogenic potential of these infections.

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