Physicochemical Properties of Marburg Virus: Evidence for Three Distinct Virus Strains and Their Relationship to Ebola Virus

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SUMMARY

The physicochemical and antigenic properties of three groups of Marburg (MBG) virus isolates, separated temporally and geographically, were compared to each other and to another member of the same family, Ebola (EBO) virus. Each MBG isolate contained seven virion proteins, one of which was a glycosylated surface protein. Peptide mapping of glycoproteins, nucleoproteins (NP) and viral structural protein (VP40) demonstrated extensive sequence conservation in the proteins of viruses isolated over a 13-year period, but homology was not evident in VP24. Some homology between the NPs of MBG and EBO was observed. A close antigenic relationship between MBG strains was found by radioimmunoassay but no evidence was found of antigenic cross-reactivity with EBO viruses. MBG virion proteins are produced from virus-specific monocistronic mRNA species. Five of the seven viral proteins were produced by in vitro translation of these RNAs. MBG virions contained one RNA species with an Mr of \(4.2 \times 10^6\) and virions had a density of 1.14 g/ml in potassium tartrate. Virus isolates from different outbreaks had distinct T1 oligonucleotide maps, but had approximately 95% homology in base sequence. No two geographically distinct virus pairs were more closely related to each other than to a third virus isolate. MBG viruses are thus similar to EBO viruses in morphology and other physicochemical properties and are very similar to each other in RNA and protein composition. Each of the three geographically and temporally distinct MBG virus outbreaks appears to have been due to a genetically distinguishable, but antigenically closely related virus strain. In addition, these studies confirm the belief that MBG and EBO viruses are members of the new virus family, the Filoviridae.

INTRODUCTION

Marburg (MBG) virus is an enveloped, negative-stranded RNA virus that is morphologically related to Ebola (EBO) virus (Regnery et al., 1981). Both viruses have been isolated from patients in Africa and produce severe haemorrhagic disease with mortality as high as 90% for EBO (Johnson et al., 1977) and 30% to 35% for MBG (Martini & Siegert, 1971). MBG virus was first isolated in 1967 from laboratory workers who had become ill after contact with tissues of monkeys imported into Europe from Uganda (Martini & Siegert, 1971), hence the alternative name 'green monkey disease'. The second isolation of MBG was from patients in a small outbreak in 1975 in South Africa (Gear et al., 1975). The third isolation of the virus was in 1980 from a physician who had treated a fatal case of the disease in a Nairobi, Kenya hospital.
Table 1. Known occurrence of MBG virus disease

<table>
<thead>
<tr>
<th>Location</th>
<th>Year</th>
<th>Cases</th>
<th>Case fatality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Germany, Yugoslavia</td>
<td>1967</td>
<td>31</td>
<td>22</td>
</tr>
<tr>
<td>South Africa (Zimbabwe)</td>
<td>1975</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>Kenya</td>
<td>1980</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Kenya</td>
<td>1987</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

* Disease was contracted by contact with tissues and blood of monkeys imported from Uganda.
† The index case was apparently infected in Zimbabwe but became ill in South Africa. Two contacts in South Africa became ill.

(Morbidity and Mortality Weekly Report, 1980). In 1987 a MBG virus was isolated from a fatal case in Kenya (E. J. Johnson, personal communication). See Table 1 for summary.

Ebola virus contains seven proteins and one strand of RNA with an M_r of approximately 4.2 x 10^6 (Elliott et al., 1985; Regnery et al., 1980). Proteins are encoded by monocistronic mRNAs which are complementary to virion RNA (Sanchez & Kiley, 1987). The Zaire and Sudan EBO viruses are both genetically distinct as determined by peptide mapping and RNA fingerprinting (Buchmeier et al., 1983; Cox et al., 1983) and antigenically distinct (Richman et al., 1983). Both viruses seem to be genetically stable, at least over the period surveyed.

This report compares the basic physicochemical properties of MBG viruses from each of the three initial outbreaks; the basic properties of the three MBG viruses are also compared to those of EBO viruses. The results indicate that (i) each MBG virus outbreak was caused by an antigenically related, but genetically distinct virus strain, (ii) variations in PAGE protein profiles that are seen between EBO virus strains are not evident among MBG strains, (iii) despite the conservation between MBG and EBO virus proteins suggested by peptide mapping, antigenic relatedness was not demonstrated and (iv) no two MBG isolates appear to be more closely related to one another than to a third geographical isolate.

**METHODS**

*Cells and virus strains.* All experiments were conducted using the E6 strain of Vero cells (McCormick et al., 1982). MBG virus strains designated by location and year of isolation and original reference were: RYC and Voege (VOG) (Germany/Uganda, 1967) (Siegert et al., 1967), OZO and Hogan (HGN) (South Africa, 1975) (Gear et al., 1975) and Musoke (MUS) (Kenya, 1980) (Morbidity and Mortality Weekly Report, 1980). EBO and MBG viruses were isolated from clinical materials in Vero cells.

*Virus growth and purification.* Roller bottles (490 cm^2) containing confluent monolayers of E6 cells were infected with virus at an input m.o.i. of 10^-2 p.f.u./cell. Following a 30 min adsorption period the infected cells were incubated at 37 °C for an appropriate time. When virion RNA was to be labelled, [5,6-^3H]uridine (New England Nuclear) was added to infected cultures at a final concentration of 20 μCi/ml at the time of infection. Infected cells were incubated in Eagle's minimal essential medium containing 2% foetal calf serum. Virus was harvested, usually 5 to 7 days post-infection, when approximately 80% of the cells were infected (as determined by immunofluorescent staining). Tissue culture fluid was clarified by low speed centrifugation and virus was concentrated by the addition of polyethylene glycol as described previously for EBO virus (Kiley et al., 1980). Pelleted virus was resuspended in TNE (0.01 M-Tris-HCl pH 7.4, 0.15 M-NaCl, 2 mM-EDTA) and purified by centrifugation through 0 to 40% potassium tartrate/30 to 0% glycerol followed by 20 to 70% sucrose (Kiley et al., 1980). Virus was diluted in TNE and pelleted by high speed centrifugation.

*PAGE.* Virion proteins were usually separated on 15% polyacrylamide slab gels (Laemmli, 1970), and were visualized by staining with Coomassie Brilliant Blue.

*Peptide mapping.* After PAGE, proteins were excised from the gel, radioiodinated, digested and analysed by electrophoresis in the first dimension followed by chromatography in the second (Buchmeier et al., 1983).

*Radioimmunoassay (RIA).* Reactivity of antisera was determined by use of a sensitive RIA procedure (Richman et al., 1983). Gamma-irradiated (2 x 10^6 rad) infected cells served as the source of virus antigen (Elliott et al., 1982).

*Isolation of messenger RNA.* Virus-specific mRNA was isolated and translated in vitro as previously described (Sanchez & Kiley, 1987).
Agarose gel electrophoresis. RNA from purified virions was obtained by the phenol–chloroform-proteinase K method (Cox et al., 1983). RNA was analysed on 1% low melting temperature agarose gels (Bethesda Research Laboratories) after denaturation with 10 mM-methylmercuric hydroxide (Bailey & Davidson, 1976).

Oligonucleotide mapping. Extracted virion RNA was digested with ribonuclease T1, labelled at the 5' end with [γ-32P]ATP and analysed on two-dimensional gels (Cox et al., 1983).

RESULTS

Virion density

The density of the VOG strain of MBG virus, as determined by centrifugation through a 10 to 40% potassium tartrate gradient, was 1.14 g/ml, approximately the same value as determined for EBO virus (data not shown). Electron microscopy of MBG virus particles purified in this manner (data not shown) demonstrated filamentous virions that were morphologically identical to those previously described for MBG and EBO viruses (Murphy et al., 1978).

PAGE analysis of proteins isolated from several MBG virus strains

Although all EBO virus isolates have similar PAGE protein profiles, Mr values of analogous proteins from different virus strains are different (Elliott et al., 1985). To determine whether this were also true for the different MBG isolates, three geographically and temporally distinct MBG virus isolates were compared by SDS–PAGE analysis. The protein mobilities of each of these isolates were indistinguishable (Fig. 1 a). In contrast, PAGE profiles of MBG and EBO virus are quite distinct (Fig. 1 b). The PAGE profiles shown in Fig. 1 clearly demonstrate the presence of five major protein bands in each MBG preparation. The large (L) protein and the glycoprotein (GP) are not always evident in stained preparations but are detectable in isotopically labelled preparations (data not shown). The seven MBG virion proteins are L (180K), GP (140K), nucleoprotein (NP), 40K structural protein (VP40), VP35, VP30 and VP24. By analogy to EBO
virus, the MBG VP35 may be a transcriptase component loosely associated with the ribonucleoprotein and the VP30 may be a second nucleoprotein. The functions of the two remaining proteins have not yet been determined but they are named to correspond to the EBO protein nomenclature system (Elliott et al., 1985).

**MBG GP is located at the virion surface**

Because the GP was not detectable in stained gels, we labelled virions with $[^3]$H]leucine and analysed the proteins by SDS–PAGE (Fig. 2). Six virion proteins were labelled. Digestion with the proteolytic enzyme bromelain removed the 140K protein from the virion demonstrating that
**Physicochemical properties of Marburg virus**

Table 2. MBG virus proteins

<table>
<thead>
<tr>
<th>$M_r$*</th>
<th>Original designation</th>
<th>Percentage of virion protein</th>
<th>$M_r$ of protein in virion (x 10$^{-6}$)</th>
<th>Number of molecules per virion</th>
<th>Current designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>180K</td>
<td>–</td>
<td>2-8</td>
<td>10-6</td>
<td>56</td>
<td>L</td>
</tr>
<tr>
<td>140K</td>
<td>VP1</td>
<td>2-0</td>
<td>7-6</td>
<td>54</td>
<td>GP</td>
</tr>
<tr>
<td>96K</td>
<td>VP2</td>
<td>27-0</td>
<td>102-0</td>
<td>1062</td>
<td>NP</td>
</tr>
<tr>
<td>38K</td>
<td>VP3</td>
<td>39-0</td>
<td>147-4</td>
<td>4594</td>
<td>VP40</td>
</tr>
<tr>
<td>32K</td>
<td>–</td>
<td>17-0</td>
<td>64-2</td>
<td>2140</td>
<td>VP35</td>
</tr>
<tr>
<td>28K</td>
<td>–</td>
<td>6-0</td>
<td>22-7</td>
<td>837</td>
<td>VP30</td>
</tr>
<tr>
<td>24K</td>
<td>VP4</td>
<td>6-2</td>
<td>23-4</td>
<td>1400</td>
<td>VP24</td>
</tr>
</tbody>
</table>

* MBG and EBO virions have similar protein PAGE profiles and $M_r$ values vary from type to type. Protein designations identify analogous proteins.

it is a surface glycoprotein. Mobilities of the remaining virion polypeptides were unchanged, suggesting that these were internally localized proteins. The GP in virions is readily labelled with [$^{3}$H]glucosamine (data not shown).

**MBG virion proteins are produced from monocistronic mRNA species**

To determine whether MBG proteins were synthesized in a manner analogous to that for EBO virus, RNA from MBG-infected cells was used to programme *in vitro* protein synthesis using a rabbit reticulocyte lysate system. Fig. 3 demonstrates the synthesis of five MBG virus-specific proteins, i.e. NP, VP40, VP35, VP30 and VP24. These proteins are analogous to previously described EBO virion proteins (Sanchez & Kiley, 1987). Neither L nor GP was detected. Fig. 3 also shows that the mRNAs are polyadenylated, a result reported previously for EBO virus (Sanchez & Kiley, 1987).

**Relative abundance of MBG virion proteins**

From the data in Fig. 1 the relative quantity of each viral protein was measured by densitometry of the gels stained with Coomassie Brilliant Blue. We determined that the RNA constitutes approximately 1.1% of the virion weight, based on the relative mass of RNA and proteins in the virion and knowing the $M_r$ of the RNA. Neglecting the lipid and carbohydrate contribution, we calculated the total mass of protein in the virion and the number of molecules per virion of each virus protein (Table 2). Similar results were obtained when isotope distribution in virions labelled with amino acid mixtures was used to determine the relative abundance of proteins (data not shown). The number of molecules of GP was probably underestimated because this protein seems to be highly glycosylated and its $M_r$ as determined by SDS–PAGE may be significantly higher than the actual value. *In vitro* translation of EBO GP mRNA produced a 70K protein (Sanchez & Kiley, 1987) while the glycosylated form of GP in the virion had an apparent $M_r$ of 125K. The MBG 140K protein, therefore, is thought to be the surface GP, although no peptide analogous to the EBO backbone GP was detected when MBG-specific mRNA was translated *in vitro* (Fig. 3). The 96K protein is the major virus NP. No function has yet been assigned to the other virion proteins although VP24 may be analogous to the membrane (M) protein of other negative-stranded viruses. The VP30 seems to be tightly bound to the virion nucleocapsid as in EBO virus and may represent a second nucleocapsid structural protein (Elliott *et al*., 1985).

**Peptide mapping shows conservation and divergence among MBG virus proteins**

Proteins from all of the MBG strains tested were the same size by SDS–PAGE. Four were excised from gels and their tryptic peptides analysed (Fig. 4). Despite isolation over a 13-year period there was remarkable homogeneity among the four proteins tested. Only the VP24 differed significantly between strains. We found only a single peptide (Fig. 4, arrow) of the viral GP which was reproducibly present in the VOG maps but absent in the MUS map. Because the NP shows a high degree of conservation and because it is the major virion nucleocapsid protein
Fig. 4. Peptide mapping analysis of selected proteins from two MBG virus strains. Proteins from the VOG (a to d) and MUS (e to h) MBG virus strains were excised from gels, radiiodinated and digested with trypsin. Peptides were analysed by electrophoresis in the first dimension and chromatography in the second. (a, e) GP; (b, f) VP; (c, g) VP40; (d, h) VP24. Only the VP24 shows significant difference in peptides. There is one peptide (arrow) in the GP which is present in the VOG maps but absent in the MUS maps.

we compared the NP peptide maps of the VOG MBG strain with those of the two EBO virus strains. We mapped single and mixed digests of VOG and Sudan and Zaire EBO virus strains searching for evidence of peptide homology (Fig. 5). We observed similarity in the distribution of peptides derived from the NPs of these viruses, and analysis of mixtures of these digests in all combinations showed that at least two peptides (Fig. 5, arrows) comigrated. No evidence exists for a NP ‘group’ antigen with these viruses. Peptide maps of viruses from the same MBG outbreak were identical.

**Antigenic cross-reactivity between MBG strains and lack of cross-reactivity between EBO and MBG**

After finding evidence of conservation between MBG and EBO virus polypeptides we examined their antigenic relatedness using a sensitive RIA that showed the presence of distinct EBO virus serotypes (Richman et al., 1983). The results (Table 3) indicated close antigenic relationship between the two MBG strains tested; however, we were unable to demonstrate any cross-reactivity between MBG and EBO viruses.

**Virion RNA species**

$[^3]H$Uridine-labelled virion RNA was extracted from representative MBG isolates and from the MAY strain of EBO and electrophoresed under denaturing conditions through a 1% agarose gel. The $M_r$ of virion RNA from each of the MBG isolates was about $4.2 \times 10^6$ (data not shown).

**$T_1$ oligonucleotide mapping of viral RNA**

We examined the genetic relatedness of different strains of MBG virus by comparing $T_1$ oligonucleotide maps of virion RNA. No differences could be detected in the individual RNA
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Fig. 5. Comparison of nucleoproteins of MBG (a), Sudan EBO (b) and Zaire EBO (c) strains by peptide mapping. NP proteins of the three strains were excised from gels and examined as in Fig. 4. Digests were examined as single maps (a, b, c) or as mixtures: MBG + Sudan (d), MBG + Zaire (e), Sudan + Zaire (f). The arrows indicate two spots conserved among the three strains.

Table 3. RIA antibody titre of human and guinea-pig antisera generated against two strains of MBG virus using each of the two strains and an EBO virus strain as antigens

<table>
<thead>
<tr>
<th>Antiserum (strain)</th>
<th>EBO (MAY)</th>
<th>MBG (VOG)</th>
<th>MBG (MUS)</th>
<th>Ratio of VOG : MUS titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (VOG)</td>
<td>&lt; 10</td>
<td>800</td>
<td>1000</td>
<td>0.80</td>
</tr>
<tr>
<td>Human (MUS)</td>
<td>&lt; 10</td>
<td>4000</td>
<td>5000</td>
<td>0.80</td>
</tr>
<tr>
<td>Guinea-pig (VOG)</td>
<td>&lt; 10</td>
<td>1500</td>
<td>1800</td>
<td>0.83</td>
</tr>
</tbody>
</table>

patterns between two viruses isolated during the 1967 outbreak (Fig. 6a and b) or in a mixture of the two RNAs (Fig. 6c). Similar results were obtained for two 1975 virus isolates from the same outbreak (data not shown). However, when representative isolates from the outbreaks of 1967, 1975 and 1980 were compared, considerable genetic heterogeneity was observed (Fig. 6a, d, e). The degree of heterogeneity of these virus strains was further assessed by co-electrophoresis of mixtures of pairs of RNAs from viruses isolated in different years (Fig. 6f, 7a, b, c). When a quantitative estimate of the relatedness of virus pairs was made by counting oligonucleotides shared by the two viruses and specific for each virus (number of common spots/total number of spots), an approximately equal degree of heterogeneity was seen for each of the three pairs of viruses (Table 4).
Fig. 6. Virion RNA from MBG viruses representing each virus outbreak was digested with T1 ribonuclease and the resulting oligonucleotides were labelled at the 5' end. These were then analysed, either singly or in pairs, by the two-dimensional polyacrylamide method described in Methods. The strains tested are as follows: (a) VOG (1967), (b) RYC (1967), (c) VOG plus RYC, (d) HGN (1975), (e) MUS (1980) and (f) VOG plus MUS.

Table 4. Relationship of MBG isolates as determined by oligonucleotide mapping

<table>
<thead>
<tr>
<th>RNA mixture</th>
<th>Common spots/total spots</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOG/HGN</td>
<td>63/114</td>
<td>55</td>
</tr>
<tr>
<td>VOG/MUS</td>
<td>60/116</td>
<td>52</td>
</tr>
<tr>
<td>MUS/HGN</td>
<td>48/98</td>
<td>49</td>
</tr>
</tbody>
</table>

**DISCUSSION**

We have previously presented data on the physicochemical properties of EBO viruses demonstrating that the virus is a negative strand RNA virus of unique morphology (very long particles with some branching) containing a monopartite RNA genome of \( M_r 4.2 \times 10^6 \) and seven virion proteins (Kiley et al., 1980; Regnery et al., 1981; Elliott et al., 1985). Studies including biological characterization, peptide mapping, oligonucleotide mapping and RIA demonstrated at least two EBO virus biotypes or strains (McCormick et al., 1983; Richman et
Fig. 7. Diagrammatic representation of T1 oligonucleotide mapping experiments where oligonucleotides of two strains were mixed before electrophoresis. A mixture of (a) VOG plus HGN, (b) HGN plus MUS and (c) VOG plus MUS. The black spots in each panel represent oligonucleotides common to both viruses while oligonucleotide spots unique to each virus are labelled with the first letter of the virus strain. H, oligonucleotides unique to HGN isolate; V, oligonucleotides unique to VOG isolate; M, oligonucleotides unique to MUS isolate. These data were used in the comparison of virus strains presented in Table 4.
al., 1983; Buchmeier et al., 1983; Cox et al., 1983). We have now described molecular and antigenic properties of three separate isolates of MBG virus, and compared these properties to those of Ebola virus.

MBG and EBO viruses share a common morphology and are similar in density (Kiley et al., 1980), PAGE profile (Elliott et al., 1985), Mr of virion RNA and synthesis of proteins via monocistronic mRNAs (Sanchez & Kiley, 1987).

There were no differences in SDS–PAGE profiles among the different MBG isolates. Peptide maps of three of the MBG virus proteins demonstrated significant polypeptide homology between strains isolated 13 years apart; however, homology was not evident in VP24. The antigenic homogeneity of MBG isolates is further supported by failure to distinguish them with a polyclonal serum, in contrast to results previously reported for EBO virus (Richman et al., 1983).

Similar conclusions apply to the analysis of the RNA from these isolates. Extensive sequence homology at the 3' end of the virion RNA from all three strains of MBG virus has been demonstrated; the base sequence of the first 70 bases of each isolate is identical (Kiley et al., 1986). Virion RNA with an Mr of 4·2 × 10^6, as found in MBG and EBO viruses, is sufficient in size to code for the known virus proteins and mRNA species for five of seven virion proteins have been identified (Fig. 3).

T1 oligonucleotide maps of the three different MBG RNAs indicated that each outbreak of the disease was caused by a genetically distinguishable virus. Approximately 50% oligonucleotide homology was observed between each of the three pairs of viruses examined, suggesting that no two isolates were more closely related to each other than to the third isolate. Fifty percent oligonucleotide homology represents approximately 95% base sequence homology (Aaronson et al., 1982). This observation of significant homology is also borne out by our recent findings of absolute base sequence conservation at the 3' end of MBG virus RNA from the 1967, 1975 and 1980 isolates (Kiley et al., 1986). Until recently we have not been able to examine the genetic stability of the MBG virus genome, since multiple isolates separated by time had not been made from any location, nor had sequential isolates from individuals been obtained.

The 1987 MBG isolate was from an individual who apparently was infected near the same location as the 1980 case, and so this virus strain may offer us an opportunity to examine MBG viruses from the same source isolated 7 years apart. Studies with the new isolate are currently underway.

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REFERENCES


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