Ebola Virus: Identification of Virion Structural Proteins

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SUMMARY

Polyacrylamide gel electrophoresis of purified Ebola virus revealed the presence of four major virion structural proteins which we have designated VP1, VP2, VP3 and VP4. Vesicular stomatitis virus (VSV) proteins were used as mol. wt. markers, and the virion proteins were found to have mol. wt. of 125,000 (VP1), 104,000 (VP2), 40,000 (VP3) and 26,000 (VP4). VP1 was labelled with glucosamine and is probably a glycoprotein. The density of the Ebola virion was approx. 1.14 g/ml in potassium tartrate. Virus nucleocapsids with a density of 1.32 g/ml in caesium chloride were released when virions were treated with detergents. Proteins VP2 and VP3 were consistently associated with released nucleocapsids and are probably the major structural nucleocapsid proteins analogous to the N protein of VSV. Protein VP4 was reduced or absent in released nucleocapsids and is probably analogous to the membrane (M) protein of VSV and similar viruses. The glycoprotein (VP1) is larger than the glycoprotein of any known negative-strand RNA virus and is not labelled well with 35S-methionine. VP1 is solubilized by detergent treatment, suggesting that it is a component of the virion spikes and analogous to the G protein of VSV. Our results, in conjunction with analysis of Ebola virion RNA (Regnery et al. 1980), strongly suggest that the virus is a negative-strand RNA virus and, along with Marburg virus, may constitute a new taxon within this group.

INTRODUCTION

Ebola virus, the aetiological agent of an acute haemorrhagic fever of man, was first isolated in northern Zaire and southern Sudan in 1977 (Bowen et al. 1977; Johnson et al. 1977). Initial characterization of the agent indicated that its morphology is very similar to, if not identical with, that of Marburg virus, which was first isolated in 1967 (Siegert et al. 1967; Bowen et al. 1977; Pattyn et al. 1977). Although the two viruses are very similar in morphology, they do not appear to be related antigenically, at least as measured by the indirect fluorescent antibody test (Johnson et al. 1977; Webb et al. 1978). To characterize these agents further we have begun a programme to delineate the chemical composition of the viruses and to establish structure–function relationships of the various virus components. This information may shed light on the mechanism of virus pathogenesis and aid in the development of a rapid, safe and sensitive diagnostic test.

The present report concerns the purification and preliminary biochemical characterization of one of the Zaire strains of Ebola virus. These studies were all conducted in the recently opened Maximum Containment Laboratory at the Center for Disease Control.
METHODS

Cell cultures. The continuous line of green monkey kidney cells (Vero; Yasumura & Kawatika, 1963) was used to prepare stock virus and for experimental procedures. Cells were grown in minimal essential medium (MEM) containing 10% heat-inactivated foetal calf serum (FCS) and were maintained on the same medium containing 2% FCS. SW13 cells, derived from a human adenocarcinoma, were obtained from the American Type Culture Collection. They were grown and maintained on the same media used for Vero cells.

Virus source and assay. The Mayinga strain of Ebola virus, which was isolated from a patient in Zaire, was used throughout. To obtain the experimental pool, virus was passaged twice in Vero cells and assayed for infectivity in the following manner. Serial 10-fold dilutions of virus were each inoculated into four test tube cultures of Vero cells. At 7 days p.i., medium was removed and cells were scraped and fixed on to Teflon-coated slides with acetone. The presence of virus antigen was determined by the indirect immunofluorescent technique described previously (Wulff & Lange, 1975). Fifty % endpoints were determined by the method of Reed & Muench (1938). The titre of the experimental pool was $10^{6.5}$ TCID50/ml.

Plaque assay. Ebola plaque assays were done in SW13 cells. Monolayer cultures in 6-well panels (Falcon) were infected in duplicate with serial 10-fold dilutions of virus. Infection inoculum was in a vol. of 0.1 ml. After a 30 min adsorption period, cells were overlaid with 4 ml of Eagle's basal medium (BME) containing 1% FCS and 1% agarose. On day 5, 1.5 ml of Hanks' balanced salt solution containing 1% agarose and 1 mg/ml neutral red were added to each well. Pinpoint plaques were seen on day 6 and were easily counted on day 7.

Infection of cells. Confluent monolayers of Vero cells in 490 cm² roller bottles were infected with a 5 ml suspension of virus in MEM-2% FCS. The input m.o.i. was approx. $10^{-8}$ TCID50/cell, and adsorption was for 1 h. After adsorption, fresh maintenance medium was added and cells were incubated at 36 °C.

Isotopic labelling of virus. At an appropriate time after infection, the culture medium was removed and replaced with medium containing either 35S-methionine, 3H- or 14C-amino acid or 3H-glucosamine. The medium for 35S-methionine labelling was MEM minus unlabelled methionine, for 3H-amino acid labelling was MEM with 10% of the normal amino acids and for 3H-glucosamine labelling was MEM with one-half the normal glucose concentration. Normal FCS was replaced with dialysed FCS when radioactive amino acids were used for labelling. Exact times of labelling are included in figure legends.

Purification of virus. Virus was purified by a modification of the technique described by Obijeski et al. (1974). At an appropriate time after isotopes were added, culture fluid was removed and clarified by centrifugation at 6500 g for 5 min at 4 °C. To this clarified solution NaCl and polyethylene glycol 6000 (PEG) were added at the rate of 23 g/l and 70 g/l, respectively. This solution was stirred slowly overnight at 4 °C and then centrifuged at 10000 g for 30 min in a Beckman J-21 centrifuge. The resultant pellet was resuspended in TSE (0.01 M-tris, pH 7.4, 0.15 M-NaCl and 2 mM-EDTA) and 3 ml amounts were layered on 13 ml 0 to 50% (w/w) potassium tartrate (KT) gradients prepared in TE buffer (2 mM-tris, pH 7.4, 2 mM-EDTA). After these were centrifuged for 18 h at 90000 g and 4 °C in an SW40 rotor, an opalescent band was found about half way down the gradient, just above a layer of cellular debris. The virus band was removed, diluted 1:1 with TSE and layered on a 13 ml 20 to 70% (w/w) sucrose gradient. This gradient was centrifuged at 160000 g for 20 h at 4 °C in an SW40 rotor. The single visible gradient band was removed, diluted at least 1:10 with TSE and the virus pelleted at 160000 g for 1 h at 4 °C. The virus pellet was resuspended in a small volume of 0.01 M-PO4 buffer and stored at -70 °C.
**Ebola virion proteins**

*Polyacrylamide gel electrophoresis* (PAGE). Virion proteins were analysed by the continuous SDS–phosphate gel system described by Maizel (1970). Samples of purified labelled virus were made 2.5% (w/v) with respect to SDS and 5% (v/v) with respect to 2-mercaptoethanol. Samples were then boiled for 2 min and sucrose and bromophenol blue were added to final concentrations of 10% and 0.001%, respectively. Samples (usually 100 µl) were then loaded on 7.5% cylindrical acrylamide gels containing 0.2% SDS and 1 M-urea. The electrode buffer was 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.1% SDS and 1 M-urea. Electrophoresis was usually at 3.5 mA/gel for 20 h.

After electrophoresis, gels were extruded from the tubes and fixed in 20% trichloroacetic acid for 24 h. This solution was decanted and replaced with 7% acetic acid. At this time the test tubes containing the gels were sealed and removed from the laboratory through a dunk tank containing a 10% sodium hypochlorite solution. The gels were then removed from the test tubes, frozen and sliced into 1 mm sections with a Mickle gel slicer (Mickle Laboratory Engineering, Co., Guildford, U.K.). Slices were placed in a vial with 6 ml of a xylene-based counting fluid containing 4% TS-1 solubilizer and were incubated overnight at 37°C. Radioactivity of each sample was determined with a liquid scintillation counter.

**Release of virion internal structure.** To release virus cores, labelled virions were treated with Triton X-100 and 1 M-KCl as described by Emerson & Wagner (1972). The volume of this mixture was adjusted to 0.5 ml with TSE and this sample was mixed with 4.5 ml of a solution containing 5.5 g CsCl per 15 ml of TSE. These gradients were then centrifuged to equilibrium for at least 24 h at 150000 g at 4°C in an SW50.1 rotor. After centrifugation, gradients were fractionated and acid-insoluble radioactivity of a sample of each fraction was determined as described previously (Kiley et al. 1974). The density of each fraction was determined by measuring its refractive index with an Abbé refractometer.

**Reagents.** Uniformly labelled 1-14C- or 3H-amino acid mixtures, 3H-uridine (20 Ci/mmol), 3H-glucosamine (86 mCi/mg) and 35S-methionine (559 Ci/mmol) were obtained from New England Nuclear, Boston, Mass., U.S.A. Caesium chloride, density gradient grade, was obtained from BDH, Poole, U.K. Density gradient grade sucrose was obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A. Triton X-100 was obtained from Eastman Chemicals, Rochester, N.Y., U.S.A. and TS-1 solubilizer was purchased from Research Products International, Elk Grove, Ill., U.S.A.

**RESULTS**

**Virus growth and purification**

When roller-bottle cultures of Vero cells were infected with Ebola virus at an m.o.i. of approx. 10⁻³, progeny virus was detected within 24 h. Virus production continued for at least 9 days with a peak titre of approx. 10⁶.⁵ TCID₅₀/ml being reached on day 4 and continuing at that level until day 9. Polyethylene glycol precipitation of virus from supernatant fluids resulted in approx. 100-fold concentration of infectivity. To purify Ebola virions and to determine their density, PEG-precipitated ³⁵S-methionine-labelled virions were banded in a 0 to 50% KT gradient. Electron microscopic examination of this band demonstrated typical Ebola virus morphology as previously described (Murphy et al. 1978). The infectivity in this band, as determined by plaque titration, was ~ 10¹⁰ p.f.u./ml. This virus band was then diluted and centrifuged to equilibrium in a 20 to 70% sucrose gradient. The virus was then pelleted and recentrifuged on a 0 to 50% KT gradient for density determination. The results of recentrifuging purified virions in a KT gradient are shown in Fig. 1. It can be seen that the maximum peak of radioactivity was recovered at a buoyant density of about 1.14 g/ml, approx. the same density at which vesicular stomatitis virus (VSV) was recovered. Cell supernates from uninfected cells did not produce any bands similar to the virus bands when carried through this purification procedure.
Fig. 1. Potassium tartrate equilibrium sedimentation analysis of $^{35}$S-Ebola and $^3$H-VSV virions. Fractions were assayed for TCA-precipitable radioactivity and refractive index. $\bullet\bullet$, $^{35}$S-methionine; $\bigcirc\bigcirc$, $^3$H-amino acids; $\blacktriangle$, density.

Fig. 2. (a) Polyacrylamide gel electrophoresis of $^{35}$S-methionine-labelled Ebola virus and (b) co-electrophoresis of $^3$H-amino acid-labelled Ebola ($\bigcirc\bigcirc$) and $^{35}$S-methionine-labelled VSV ($\bullet\bullet$).

**Virion polypeptides**

To determine the nature of the Ebola virion structural polypeptides, purified virions labelled with either a $^3$H-amino acid mixture, or $^{35}$S-methionine were treated with SDS and mercaptoethanol and analysed on 7.5% polyacrylamide gels. The electropherogram in Fig. 2(a) illustrates the polypeptide pattern observed when virions were purified from the supernatant fluid of infected cells grown in the presence of $^{35}$S-methionine from day 2 to day 5 p.i. The profile demonstrates three major polypeptides (fractions 33, 74, and 95) and two minor polypeptides (fractions 10 and 82). The minor polypeptides were not consistently present and may represent aggregates or breakdown products. The purification procedures have apparently eliminated most host cell proteins since the background is quite low. Electrophoresis of PEG-pelleted unpurified virus (data not shown) showed significant...
radioactivity near the top of the gel and approx. 10 peaks of radioactivity elsewhere in the
gel. When virions were grown in the presence of \(^{3}H\)-amino acids and co-electrophoresed
with \(^{35}S\)-methionine-labelled VSV the results depicted in Fig. 2(b) were obtained. An
additional polypeptide not seen in \(^{35}S\)-labelled virions was evident at fraction 20 in this
figure. We have designated the four major proteins as VP\,1 to VP\,4, with VP\,1 being the
slowest migrating protein and VP\,4 the fastest migrating protein.

\textit{Mol. wt. estimations of virus polypeptides}

The approximate mol. wt. of Ebola virion polypeptides was estimated by the procedure
used in our calculations were those reported by Obijeski \textit{et al.} (1974), namely, 160,000 for
the L protein, 65,000 for the G protein, 54,000 for the N protein, 42,000 for the NS protein
and 27,000 for the M protein. In our gel system a plot of the logarithm of mol. wt. versus
the relative electrophoretic mobility also yielded a straight line.

Using data similar to that presented in Fig. 2(b) the mol. wt. versus relative migration
plot depicted in Fig. 3 was constructed. With VSV proteins as markers the mol. wt. of the
Ebola proteins were found to be 125,000 (VP\,1), 104,000 (VP\,2), 40,000 (VP\,3) and 26,000
(VP\,4).

\textit{Incorporation of glucosamine}

To determine if the virion contained any glycoprotein the virus was grown in the presence
of \(^{3}H\)-glucosamine and co-electrophoresed with Ebola virus labelled with \(^{14}C\)-amino acids.
The result of such an experiment is presented in Fig. 4 and demonstrates that VP\,1 is a
glycoprotein. Since this protein was not labelled in Fig. 2(a) it appears to be a glycoprotein
containing few or no methionine residues. Further experiments (data not shown) have
indicated that even when VP\,1 is heavily labelled with glucosamine, label cannot be detected
in the other virion proteins.

\textit{Release of nucleocapsids from Ebola virions}

To obtain more information as to the location of the virion proteins, virions were dis-
rupted with detergent and centrifuged to equilibrium in CsCl. Fig. 5 presents the results of
Fig. 4. Co-electrophoresis of purified Ebola virions grown in the presence of either $^3$H-glucosamine ($\bigcirc$--$\bigcirc$) or $^{14}$C-amino acids (●--●).

Fig. 5. Caesium chloride equilibrium density gradient analysis of nucleocapsids released by detergent treatment of a mixture of $^{35}$S-methionine-labelled VSV (●--●) and $^3$H-amino acid-labelled Ebola virions ($\bigcirc$--$\bigcirc$). ▲–▲, Density.

Fig. 6. Electrophoretic analysis of the polypeptides in the peak fraction (fraction 16) from the CsCl gradient in Fig. 5. ○–○, $^3$H-Ebola; ●–●, $^{35}$S-VSV.
an experiment in which Ebola virions labelled with \(^3\)H-amino acids were mixed with \(^35\)S-
methionine-labelled VSV treated with Triton X-100-KCl and then centrifuged to equili-
brum in a CsCl gradient. This treatment released a structure with a density of approx.
1.32 g/ml from both viruses.

To determine the polypeptide composition of these 1.32 g/ml virus nucleocapsid struc-
tures an aliquot of the \(^3\)H-Ebola-\(^35\)S-VSV virion mixture depicted in Fig. 2(b) was detergent
treated and centrifuged to equilibrium in CsCl as in Fig. 5. Peak fractions from the resultant
1.32 g/ml peak were pooled, concentrated by TCA and electrophoresed. The results
presented in Fig. 6 indicate that the structure contains only the N protein of VSV and the
VP2 and VP3 proteins of Ebola. The nature or significance of the \(^3\)H peak in fraction 16
has not yet been determined. It may represent a large protein analogous to the L protein
of VSV or it may be an aggregate. If it were analogous to the L protein of VSV it should not be
present with the nucleocapsids after detergent–high salt treatment (Kiley & Wagner, 1972).

Discussion
Because Ebola virus (and Marburg virus) closely resembles the rhabdovirus group in
structure (Murphy et al. 1978), it seems appropriate to discuss our findings in relation to
members of this and related virus groups.

We have determined that the Ebola virion contains four major proteins which we have
designated VP1, VP2, VP3 and VP4. Two additional minor peaks of radioactivity appeared
inconsistently and have not been conclusively identified as virion structural proteins. They
may be aggregates or breakdown products. By using VSV virion proteins as markers, the
mol. wt. of the major proteins were found to be 125000, 104000, 40000 and 26000, respect-
ively. The largest protein (VP1) was the only one labelled by glucosamine.

The two major proteins present in the virion are VP2 and VP3. When virions were
detergent-disrupted, these two proteins consistently remained associated with the presumed
virion nucleocapsid which had a density of 1.32 g/ml in CsCl. One or both of these proteins
may be analogous to the N protein of VSV, which is the major virion protein and is the
structural protein that is tightly complexed with virus RNA to form the ribonucleoprotein
(Kang & Prevec, 1969; Wagner et al. 1969). Alternatively, the presence of two proteins
tightly bound to the virus RNA may be analogous to the situation with paramyxoviruses,
in which the nucleocapsid generally contains a protein that has a mol. wt. of about 60000
but is susceptible to some proteolytic enzymes. Under certain conditions this nucleocapsid
protein is cleaved to form a protein with a mol. wt. of 45000 (Mountcastle et al. 1970);
thus both cleaved and uncleaved forms may be present in a virus population.

Other studies with paramyxoviruses suggest that a second non-glycosylated protein
may be bound to the nucleocapsid and that the avidity of this binding may vary among
the different viruses studied (Mountcastle et al. 1971; Stone et al. 1972). Tryptic peptide analysis
of Ebola proteins VP2 and VP3 should determine whether they are two distinct proteins or
they share a precursor-product relationship.

Protein VP4 may be analogous to the M protein of the rhabdovirus group (Wagner
et al. 1972). It is non-glycosylated and has a mol. wt. of approx. 26000. When virions are
disrupted by treatment with Triton X 100-KCl, this protein is solubilized, which indicates
that it is present in the virion envelope. A similar protein of approx. mol. wt. 40000 is also
present in the envelope of paramyxoviruses (Scheid & Choppin, 1973).

The protein which we have designated VP1 is a large glycosylated protein with a mol. wt.
of approx. 125000. Since rhabdoviruses and other members of the negative-strand viruses
contain an envelope glycoprotein (Obijeski et al. 1974; Choppin & Compans, 1975; Compans
& Choppin, 1975; Wagner, 1975), the presence of this glycoprotein in Ebola virus is not
surprising. By analogy with other members of these virus groups, it is most likely the structural component (G protein) of the projections found on the surface of the Ebola virion (Murphy et al. 1978). Because Ebola is similar in protein composition to the negative-strand viruses the apparent size of this protein is surprising. The largest rhabdovirus glycoprotein has a mol. wt. of approx. 78,000 (Knudson & MacLeod, 1972) and the average mol. wt. of the rhabdovirus G proteins is about 68,000 (Wagner, 1975). The HN glycoprotein of Newcastle disease virus has a mol. wt. of approx. 74,000 (Mountcastle et al. 1971) and the uncleaved haemagglutinin (HA) glycoprotein of influenza virions may be as large as 80,000 (Skehel & Schild, 1971). Members of the Bunyaviridae, which are probably negative-strand viruses, do possess a glycoprotein of approx. the same mol. wt. as VP1; but they also contain another smaller glycoprotein, and the single virion nucleocapsid protein has a mol. wt. of approx. 25,000 (Obijeski & Murphy, 1977). The virions of this group also contain three species of RNA with an aggregate mol. wt. of approx. $5 \times 10^6$ whereas Ebola virus contains only one RNA species.

The fact that Ebola virions contain only a single negative-strand RNA species (Regnery et al. 1980) and the proteins described above indicates that Ebola virus is probably a member of the negative-strand RNA group of viruses and closely resembles the rhabdoviruses in certain morphological aspects and in general protein composition. Whether Ebola and the structurally similar Marburg virus are charter members of a new taxon or are rather bizarre members of the Rhabdoviridae family has yet to be determined. We would predict that although certain fish viruses (McAllister & Wagner, 1975) and rabies virus (Sokol et al. 1971) are included in the rhabdovirus group despite lack of NS protein and presence of two M proteins, Ebola and Marburg viruses are sufficiently different both morphologically and chemically to warrant placement in a new taxon.

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REFERENCES


Ebola virion proteins


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