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Antigenicity and Vaccine Potential of Marburg Virus Glycoprotein Expressed by Baculovirus Recombinants

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There is no effective vaccine for Marburg virus (MBGV) or any other filovirus, nor enough pertinent information to expedite rational vaccine development. To ascertain some of the minimal requirements for a MBGV vaccine, we determined whether whole inactivated MBGV, or a baculovirus-expressed virion subunit, could be used to immunize guinea pigs against a lethal infection. Baculovirus recombinants were made to express the MBGV glycoprotein (GP) either as a full-length, cell-associated molecule or a slightly truncated (5.4%) product secreted into medium; the latter, for its far greater ease in manipulation, was tested for its vaccine potential. Like MBGV GP, both the full-length and truncated GP expressed by baculovirus recombinants were abundantly glycosylated with both N- and O-linked glycans; differences in glycosylation were detectable, but these could not be shown to affect antigenicity with respect to available antibodies. The recombinant truncated glycoprotein elicited protection against lethal challenge with the MBGV isolate from which it was constructed and less effectively against an antigenically disparate MBGV isolate. Killed (irradiated) MBGV antigen was protective, in a reciprocal fashion, against both MBGV types. In a preliminary assessment of possible protective mechanisms, serum antibodies from immune animals were shown to be sufficient for protecting naive guinea pigs from lethal MBGV infection.

INTRODUCTION

Marburg virus (MBGV), a member of the virus family Filoviridae, causes acute hemorrhagic fever with high mortality rates in both human and nonhuman primates. The first recognized infection of humans by MBGV occurred in 1967 when simultaneous outbreaks of hemorrhagic fever occurred in Marburg and Frankfurt, Germany, and in Belgrade, Yugoslavia (Martini and Siegert, 1971). All cases were associated with laboratory workers engaged in processing kidneys from African green monkeys for cell culture production (Smith *et al.,* 1967). Two cases of hemorrhagic fever caused by a virus similar to the 1967 MBGV were identified in 1980 in Kenya (Smith *et al.,* 1982). In 1987 MBGV was isolated from a fatal case in Kenya, and was subsequently shown to differ substantially from the original isolate (Johnson *et al.,* 1996). In all occurrences, infected individuals suffered severe illnesses with mortality rates more than 28%.

The genome of MBGV consists of a single strand of RNA of minus polarity approximately 19 kb long. This RNA encodes 7 proteins, all of which are found in the virion. Gene order is 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′ and is similar to that of other members of the virus order Mononegavirales (which also include Paramyxoviridae and Rhabdoviridae) (Feldmann *et al.,* 1992). Although the functions of most of the MBG viral proteins have not yet been experimentally determined, functions of the individual proteins have been proposed based upon their homology to better defined proteins found in paramyxoviruses. The three structural proteins examined were NP, GP, and VP40. NP is a nucleocapsid protein and most likely functions to encapsidate the viral RNA (Sanchez *et al.,* 1992). VP40 is the most abundant component of the virion, and most likely serves as a matrix protein, mediating interactions between the nucleoprotein complex and the lipid membrane (Feldmann *et al.,* 1993; Elliott *et al.,* 1985). GP is the viral glycoprotein, which trimerizes to form spikes, 7 nm long, on the surface of the virion (Feldmann *et al.,* 1993, 1991; Geisbert and Jahrling, 1995; Kiley *et al.,* 1988). GP remains the only known viral protein exposed on the surface of the virion and thus appeared to be an attractive target for neutralizing antibodies. GP is abundantly glycosylated with over half its apparent molecular mass (M_r 170 KDa) attributable to glycan (Feldmann *et al.,* 1991, 1994; Becker *et al.,* 1996). The MBGV genome, unlike that of Ebola virus, encodes the viral glycoprotein in a single open reading frame, resulting in the uncomplicated production of only a full-length transmembrane form of GP (Will *et al.,* 1993; Volchkov *et al.,* 1995; Sanchez *et al.,* 1996).

It is unsettling, with a lethal pathogen like MBGV, to have no efficacious vaccine or therapy, and to possess only minimal and somewhat discouraging data pertaining to successful vaccination in animals (i.e., Skripchenko *et al.,* 1994 and Ignat'ev *et al.,* 1995 reported survival after MBGV challenge of only 50% of nonhuman

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primates and 40–60% of guinea pigs previously immunized with formalin-inactivated MBGV antigen). The reassuring knowledge that outbreaks of human disease have been uncommon and self-limited is counterbalanced by an almost complete ignorance of the virus' natural host, the factors that restrict the virus to its current ecological niche, and the potential for wider spread. Currently, a vaccine for MBGV might be realistically appropriate only for health care workers in areas where the threat of MBGV disease exists, international teams most likely to respond to active outbreaks, and a small population of laboratory workers. Data reported herein suggest there are no extraordinary barriers to MBGV vaccine development, and that the single viral glycoprotein may be a sufficient protective antigen.

MATERIALS AND METHODS

Cell cultures and viruses

*Sf*9 cells (Invitrogen, San Diego, CA), derived from *Spodoptera frugiperda* ovary, were maintained in Sf900 II serum-free medium (Gibco BRL, Gaithersburg, MD) at 27°C, as were High Five cells (Invitrogen, San Diego, CA), derived from *Trichoplusia ni* egg cell homogenates. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant viruses were cultured and assayed in *Sf*9 cells according to previously published methods (O'Reilly *et al.*, 1992). Vero E6 cells (Vero C1008, ATCC CRL 1586) were grown in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). MBGV (strain Musoke) was isolated from a human case in 1980 in Kenya (Smith *et al.*, 1982). Because this virus is virulent for nonhuman primates but not rodents, it was adapted for guinea pig lethality by eight consecutive passages in strain-13 guinea pigs (inoculating subcutaneously, harvesting spleen). A virulent plaque-purified derivative was obtained from the guinea pig passage eight material, thrice plaqued in Vero E6 cells. MBGV (strain Ravn) was isolated from a fatal human case in 1987 in Kenya (Johnson *et al.*, 1996). This virus was similarly adapted for guinea pig lethality but required only two passages, and a three-time plaque-purified virulent virus was obtained from the guinea pig passage 2 material. Plaques picked from MBGV (strain Musoke), guinea pig passage 6, were variably lethal or in some cases completely nonlethal for strain-13 guinea pigs, and one of the nonlethal plaque-picked derivatives was chosen as an immunizing virus for preparation of immune serum.

Construction of the *Ac*NPV recombinant transfer vectors and recombination with *Ac*NPV

Marburg gene clone pGem-GP was generously provided by Heinz Feldmann and Anthony Sanchez (Centers for Disease Control and Prevention, Atlanta, GA) (Will *et al.*, 1993; Sanchez *et al.*, 1989). The GP gene from pGem-GP was subcloned into pBluescript-KS(+) using classical molecular biology techniques (Sambrook *et al.*, 1989). The full-length GP gene was excised from the resulting clone (pKS-GP) with *Eag*I and *Eco*RI, and this fragment ligated into the *Not*I and *Eco*RI sites of the *Ac*NPV transfer vector pVL1392. The resulting clone was designated pVL1392-GP.

A deletion mutant of GP, which resulted in a 37 amino acid carboxyl-terminal-truncated protein missing the transmembrane domain of GP, was constructed using PCR. This gene and its product were designated GPΔTM. Forward (5′-CAG<u>AAGCTT</u>CCCTAACATGAAGACC-3′) and reverse (5′-CTG<u>AAGCTT</u>ATTTACCACCCAGAC-3′) primers for GPΔTM contained *Hin*dIII cleavage sites (underlined). All PCR reactions were performed with 1 ng of pGem-GP as template DNA, 1 μg each of forward and reverse primer, and the thermostable polymerase *Pfu* (Stratagene Cloning Systems, La Jolla, CA). The reaction conditions used were: 15 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The PCR product was ligated into the *Sma*I site of pBluescript-KS(+), and positive clones were identified. The GPΔTM gene was subcloned into the *Hin*dIII site of pBluebac III.

Production of recombinant baculoviruses

Recombinant baculoviruses were generated by cotransfection of an *Ac*NPV transfer vector containing a MBGV gene insert and linear wild-type baculovirus DNA into the *Sf*9 cell line according to standard methods (O'Reilly *et al.*, 1992). Briefly, progeny in the transfection supernatant were screened by plaque assay for the occlusion-negative phenotype. The clones of recombinant virus identified as occlusion-negative were plaque purified three times in succession. After purification, clones were amplified by growth in *Sf*9 cells, cultured in serum-free medium, expanding from a 24-well plate (1×10^5 cells/well), to a T25 (3×10^6 cells/flask), followed by growth in 100 ml suspension of *Sf*9 cells (1.5×10^6 cells/ml). The titer of the final stock of recombinant baculovirus was determined by plaque assay on *Sf*9 cells.

Expression of recombinant protein in *Sf*9 cells

*Sf*9 cells grown in a 6-well plate at a density of 1×10^6 cells/ml were infected at a multiplicity of infection (m.o.i.) of 1 PFU per cell. Cells were metabolically labeled sequentially every 24 h, starting at 24 h postinfection and continuing until 72 h postinfection. For each time point, the infected cells were starved for 30 min in Sf900 II serum-free medium without cysteine or methionine, followed by labeling for 4 h in the same medium supplemented with 100 μCi/ml each of [^{35}S]methionine and [^{35}S]cysteine. After 4 h, the supernatant was removed,

mixed with an equal volume of 2× extraction buffer (100 mM Tris–Cl, pH 8.0, 200 mM NaCl, 2% NP-40, 2% Trasylol, 200 μg/ml PMSF), and stored at −70°C. The cells were lysed in 500 μl of 1× extraction buffer on ice for 5 min, and nuclei were pelleted at 800 *g* for 5 min. The resulting cytoplasmic extract was stored at −70°C. For scale-up of protein production, 50- or 100-ml suspension cultures of insect cells were infected with recombinant baculovirus at an m.o.i. of 1, and cells or medium were harvested at the peak time of expression.

Immunoprecipitation of recombinant MBGV proteins

Expression of recombinant protein was analyzed by immunoprecipitation (Schmaljohn *et al.*, 1987, 1983). Briefly, convalescent guinea pig anti-MBGV polyclonal serum was adsorbed to protein A Tris–acryl beads (Pierce Immunotechnology, Rockford, IL) in a ratio of 15 μl serum:100 μl 50% bead slurry at 4°C for 1 h with continuous rocking. Unbound antibody was removed by washing the beads once in 0.5 ml Zw wash buffer (10 mM Tris–Cl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.4% Zwittergent 3-14, 1% Trasylol, 100 μg/ml PMSF). One hundred microliters of a 50% slurry of antibody-coated beads in Zw wash buffer was mixed with 100 μl labeled sample and the suspension incubated overnight at 4°C on a rocker. Beads were washed three times in Zw wash buffer at 4°C and the final pellet was resuspended in 50 μl of disruption buffer (50 mM Tris–Cl, pH 6.8, 4% SDS, 4% β-mercaptoethanol, 10% glycerol, 1 mg/ml bromophenol blue) and heated in a boiling water bath for 5 min. Beads were pelleted and 25 μl of the resulting sample was electrophoresed on a 12.5% SDS–polyacrylamide: DATD gel.

Lectin blots for the analysis of the carbohydrate content of recombinant expressed MBGV GP and GPΔTM

The carbohydrate content of recombinant expressed MBGV GP genes and GP from purified MBGV was compared using the DIG (digoxigenin) Glycan Differentiation kit (Boehringer Mannheim, Indianapolis, IN), following the manufacturer's instructions. Briefly, 10 μl of unlabeled *Sf*9 lysate or supernatant was electrophoresed on a 12.5% SDS–polyacrylamide gel and the proteins in the gel were transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C then were incubated with lectin-DIG at room temperature for 1 h, followed by washing three times. Anti-DIG-alkaline phosphatase was incubated with each membrane for 1 h at room temperature. The membranes were washed three times and developed with a BCIP/NBT stain. Reactions were stopped by rinsing with 10 mM EDTA followed by H_2O. The following lectins were used: *Arachis hypogaea* (peanut) agglutinin (PNA), which specifically binds the unsubstituted galactose β(1-3)*N*-acetylgalactosamine cores of O-glycans; *Datura stramonium* agglutinin (DSA), which binds galactose β(1-4)glucosamine in complex or hybrid type glycans; *Galantus nivalis* agglutinin (GNA), which recognizes terminal mannose linked α(1-3), α(1-6), or α(1-2) to mannose found in *N*-glycans; *Maackia amurensis* agglutinin (MAA), which reacts specifically with α(2-3)-linked sialic acids; and *Sambucus nigra* agglutinin (SNA), which reacts with α(2-6)-linked sialic acids.

Growth and concentration of MBGV

MBGV strain Musoke or strain Ravn were grown in Vero E6 cells and, in some instances, further concentrated. Briefly, Vero E6 cells seeded into roller bottles and grown to confluency were infected with MBGV at a low m.o.i. (<0.05). On day four postinfection, the medium in each roller bottle was replaced with 40 ml of EMEM supplemented with 10% FBS. Supernatant from roller bottles was harvested at the time peak cytopathic effects were observed, typically 6–7 days postinfection. The medium was clarified (15 min, 1500 *g*) and virus concentrated by polyethylene glycol precipitation (7.5% w/w, NaCl adjusted to 0.5 M) at 4°C for 4 h. After centrifugation at 10,000 *g* for 30 min, pellets were resuspended in TNE (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, and 1 mM EDTA) overnight at 4°C. One-milliliter aliquots of the resuspended PEG pellet were layered atop 20–60% sucrose (prepared in TNE) gradients and the gradients were centrifuged at 38,000 rpm in an SW41 rotor for 4 h. The visible virus band was collected. Samples were inactivated by irradiation (6MR, ^{60}Co source) and tested for absence of infectivity in cell culture before use.

Metabolic labeling of MBGV

Vero E6 cells were infected at a high m.o.i. (5–10). After 28 h, growth medium was removed, and infected cells were starved for 30 min in MEM lacking cysteine and methionine. Cells were refed with MEM without cysteine or methionine, supplemented with 2% FBS, 100 μCi/ml [^{35}S]methionine, and 100 μCi/ml [^{35}S]cysteine. Cells were labeled for 18–20 h. Medium was removed from the cells, clarified (1500 *g*, 15 min), and virus was pelleted by centrifugation (2 h, SW28 rotor at 25,000 rpm).

Monoclonal antibodies directed against MBGV strain Musoke

Ten female Balb/C mice were immunized twice subcutaneously at one site, dorsally, near the base of the tail, on days 0 and 66, with irradiated MBGV (strain Musoke) (20–40 μg/mouse) emulsified in Freund's incomplete adjuvant. Sera obtained 12 days after the second immunization were assayed for MBGV-specific antibody by ELISA, Western blot, and radioimmunoprecipitation against purified virion. A third immunization, administered intravenously in HBSS on day 112, was given to three mice

whose sera had reacted particularly well with MBGV GP. Three days (two mice, fusions I and II) or 4 days (one mouse, fusion III) after the third immunization, spleens were taken from these three mice. Cells were fused with SP2/0 myeloma cells according to standard methods for fusion and cell husbandry; hybridomas from wells with ELISA-positive supernatants were subcloned twice by limiting dilution (Early and Osterling, 1985).

ELISA using GPΔTM or purified MBGV as antigen

Flexible PVC ELISA plates (Dynatech Laboratories, Chantilly, VA) were coated with 50 μl of antigen/well diluted in PBS and held overnight at 4°C. The antigen used to coat the plates was a 1:500 dilution of irradiated MBGV (~1 mg/ml total protein = 100 μg/ml GP) or 1:50 of GPΔTM (~10–20 μg/ml). To block nonspecific binding, 200 μl of 5% nonfat dry milk in PBS containing 0.02% Tween 20 (PBSTM) was added to each well and plates were incubated at room temperature for 1 h. Plates were washed five times with 200 μl/well PBS containing 0.02% Tween 20 (PBST). Primary antibodies were diluted in PBSTM containing 1% heat-inactivated fetal calf serum, and 50 μl of diluted antibody was added to each well. The test sera (primary antibody) was diluted down the plate in duplicate by half-log (3.16-fold) dilutions. Plates were incubated for 1 h at room temperature and washed five times with PBST. Secondary antibody was either horseradish peroxidase (HPO)-labeled goat-anti-mouse (IgM, IgG, IgA) or HPO-goat-anti-guinea pig (IgG H + L) (Cappel), diluted to 1:2000 in PBSTM containing 1% heat-inactivated fetal calf serum. Diluted secondary antibody (100 μl) was added to the appropriate wells and the plates were incubated for 1 h at room temperature. Plates were washed five times with PBST and 100 μl of 2,2′-Azinobis-[3-ethylbenzothizoline-6-sulfonic acid] diammonium salt (ABTS) substrate was added to each well. After a 30-min incubation at room temperature, the optical density of each well at 405 nm was determined. Endpoints were calculated using a four-parameter curve fit (SOFTmax software, Molecular Devices Corp.) of background subtracted mean OD versus dilution, followed by extrapolation of the dilution at which the OD was 0.20.

Immunization protocol for guinea pigs

Inbred strain 13 or outbred Hartley guinea pigs were immunized subcutaneously with antigen prepared in RIBI MPL + TDM + CWS (Monophosphoryl Lipid + Synthetic Tehalose Dicorynomycolate + Cell Wall Skeletion) emulsion (RIBI ImmunoChem Research Inc.) with a total volume of ~0.5 ml administered at two dorsal sites. Animals were anesthetized, bled, and subsequently boosted with another dose of antigen approximately 28 days after primary inoculation. A second bleed and boost was performed approximately 28 days after the first. The animals were bled 14 days later and challenged subcutaneously with 100–1000 PFU guinea pig-adapted MBGV. Animals were examined daily for signs of illness. On day 7 postinfection animals were bled for determination of plasma viremia titers. On day 14 postinfection, any surviving animals were bled and viremias assessed. Animals were followed until at least 28 days after infection, anesthetized, and exsanguinated. Viremias were determined, by direct plaque assay on Vero E6 cells (Moe *et al.,* 1981), from heparinized plasma collected on days 7 and 14 postinfection.

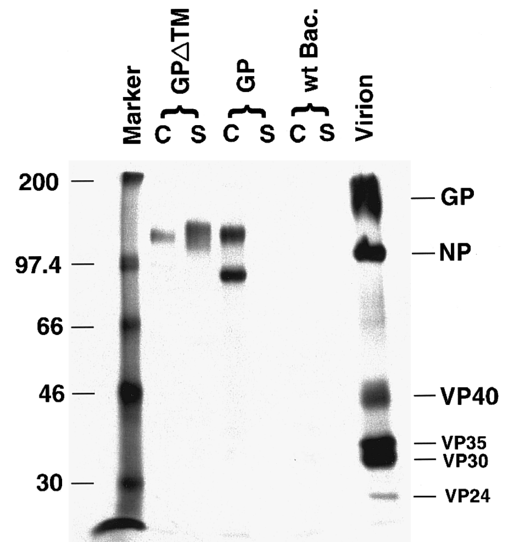

FIG. 1. Immunoprecipitation of MBG proteins expressed in Sf9 cells with convalescent guinea pig polyclonal anti-MBGV serum. Sf9 cells were infected with recombinant baculoviruses that expressed the truncated secreted Marburg glycoprotein (GPΔTM), Marburg glycoprotein (Baculo. GP), or wild-type baculovirus (wt Baculo.) at an m.o.i. of 1. At 48 h postinfection, both cell lysate (C) and supernatant (S) were collected. The samples were immunoprecipitated and analyzed by SDS–PAGE. The positions of ^{35}S-labeled MBGV (strain Musoke) structural proteins from virus grown in Vero E6 cells are shown.

Passive protection in naive guinea pigs

Sera from immune strain 13 guinea pigs were administered intraperitoneally to naive Strain 13 guinea pigs 2 h before subcutaneous inoculation with either MBGV (strain Musoke) or MBGV (strain Ravn) (1000 PFU guinea pig adapted MBGV). Animals were examined every day for signs of illness. A day 10 postinfection bleed was obtained for determination of plasma viremia.

RESULTS

Analysis of protein products expressed from recombinant baculoviruses

Protein products produced from the recombinant baculoviruses were analyzed and compared with proteins from MBGV (Fig. 1). Samples were immunoprecipitated from either cell lysates or supernatant. GPΔTM was the

TABLE 1

Reactivities of Virion-Associated and Recombinant MBGV Glycoprotein wth Lectins

	Lectin				
	GNA	DSA	PNA	MAA	SNA
Virion GP	−	+++	+++	−	−
Sf9 GP	+++	+	+++	+	−
Sf9 GPΔTM	−	−	+++	−	−

Note. +++ Indicates a very strong reactivity. + Indicates a weak signal.

only recombinant protein readily demonstrable in cell-free supernates. Both the GP and GPΔTM produced in *Sf*9 cells migrated faster than authentic GP from MBGV grown in Vero E6 cells, but considerably more slowly than their core molecular masses predicted from amino acid sequence (74.4 kDa for GP and 70.1 kDa for GPΔTM). This observation, which suggests substantial but incomplete glycosylation of GP in *Sf*9 cells compared to GP in Vero cells, is consistent with data reported previously (Becker *et al.,* 1996). Somewhat unexpectedly, GPΔTM from *Sf*9 supernatant appeared more heterogeneous in size than either GPΔTM or GP in cells, possibly indicating differential glycosylation. In addition, the GPΔTM in supernatant had an apparent maximum molecular mass greater than that of full-length GP and the GPΔTM found in cells, an observation which was reproducible in multiple experiments (data not shown). A second major product at ~86 kDa was immunoprecipitated from *Sf*9 cells expressing the recombinant GP. This product may represent either a stable breakdown product of the full-length GP or incompletely glycosylated protein. Considering that GP of MBGV is modified with both O- and N-linked oligosaccharides that contribute nearly half of the molecular mass of fully processed GP, the differences observed in the apparent molecular masses of the glycoproteins may be explained by the differential glycosylation in insect cells (*Sf*9) when compared to mammalian cells (Vero E6) (Becker *et al.,* 1996).

Carbohydrate content of baculovirus produced MBGV proteins compared with proteins found in intact virions

The extensive N- and O-linked glycosylation normally found with MBGV GP was examined using various lectins to determine the extent of glycan processing in the recombinant GP proteins compared to the virion protein (Table 1). It was clear that the three versions of GP differed in glycan content. The virion-associated GP bound both PNA and DSA as reported previously, indicating the presence of complex or hybrid glycans, as well as O-linked glycans. However, we were not able to reproduce the positive, albeit weak, binding to GNA reported by Feldmann *et al.* (1991). Surprisingly, the recombinant GPΔTM in supernatant was found only to bind PNA.

In contrast to the GPΔTM protein, the GP protein reacted strongly with GNA and PNA, and weakly with MAA and DSA. The strong reaction with GNA indicates that a significant amount of the glycan associated with the recombinant membrane-bound GP is of the high mannose type and thus represents unprocessed *N*-glycan. However, the weak reaction observed with DSA indicates that some complex N-linked glycans are present on the molecule and thus a minority of the glycans are processed. Reactivity with PNA indicates that at least some of the O-linked glycosylation sites are being utilized in the insect cells. Finally, weak reaction with MAA may indicate the presence of sialic acid residues in the recombinant GP protein that are not found in the virion-associated protein.

Reactivity of recombinant GPΔTM antigen with antibodies specific for MBGV GP

The reactivity of a subset of monoclonal antibodies (Mabs) with recombinant GPΔTM protein from *Trichoplusia ni* cells, MBGV (strain Musoke), and MBGV (strain Ravn) was examined by ELISA (Table 2). Of the GP-specific Mabs examined, all reacted equally well with MBGV (strain Musoke) antigen and recombinant GPΔTM except Mab III-5B8, which demonstrated a titer about 100-fold lower to GPΔTM antigen. Only two of the MBGV GP-specific Mabs reacted with MBGV (strain Ravn) GP (II-7G8 and III-3H10). Mabs specific for MBGV proteins other than GP did not react with recombinant GPΔTM. The NP- and VP-40-specific Mabs crossreacted well between MBGV strains Musoke and Ravn, while the two VP-30-specific Mabs did not. Experiments (not shown) involving reciprocal competitive inhibition and epitope mapping have demonstrated the GP-specific Mabs in Table 2 to react with at least six distinct epitopes, whereas the two VP-30 Mabs are thus far indistinguishable from one another.

The GPΔTM in serum-free supernatants was demonstrated to be a useful antigen for detection of anti-GP response in guinea pig serum (Fig. 2). A titration of serum from convalescent guinea pigs against both purified MBGV and GPΔTM demonstrated that a substantial portion of the antibody response in the convalescent animals was directed against GP. The interpolated endpoints in $\log_{10}$ of convalescent serum versus GPΔTM and virion were determined to be 4.02 and 4.67, respectively.

Immunization of strain 13 guinea pigs with an inactivated MBGV vaccine and *Sf*9-produced GPΔTM

Antigens used in the first experiment (four groups of 10 animals each) were: cell-free *Sf*9 supernatants con-

TABLE 2

Reactivity of Monoclonal Antibodies with MBGV and Recombinant Antigens

	Log_{10}titer of MAb[a]				
Antibody	MBGV-Musoke	MBGV-Ravn	GPΔTM	Specificity	Isotype
III-3H5	5.94	<3.0	6.09	GP	M
II-9G4	6.26	<2.0	6.38	GP	G1
III-4B11	6.13	<2.0	6.29	GP	G1
III-5E2	5.54	<2.0	5.60	GP	G1
III-5B8	5.68	<2.0	3.76	GP	G1
II-7C11	6.41	<2.0	6.37	GP	G1
III-4C9	5.28	<2.0	5.24	GP	G1
II-10D10	5.53	<2.0	5.60	GP	G1
II-7E9	5.91	<2.0	6.33	GP	M
II-7G8	5.38	5.47	5.42	GP	G3
II-9F8	6.12	<2.0	5.39	GP	G3
II-10D9	5.82	<2.0	5.77	GP	ND
III-2C12	5.57	<2.0	5.92	GP	G1
II-7E10	>6.5	<2.0	6.46	GP	M
III-2C10	5.67	<2.0	5.80	GP	G1
III-5D7	5.24	<2.0	5.30	GP	G1
III-3H10	6.38	3.93	6.18	GP	M
III-10D5	6.50	6.47	<2.0	NP	M
III-5F8	>6.5	>6.5	<2.0	NP	M
III-5F7	>6.5	>6.5	<2.0	NP	M
III-10D6	6.41	6.49	<2.0	NP	M
III-5F11	5.30	<2.0	<2.0	VP30	G1
III-5F12	5.26	<2.0	<2.0	VP30	G1
III-1H11	4.47	4.75	<2.0	VP40	G1

[a] Mab-containing ascitic fluids were precipitated with 50% saturated ammonium sulfate, dialyzed, electrophoresed, stained, analyzed by densitometry, and adjusted to 2 mg/ml Mab content based on this estimate. Endpoint was the extrapolated dilution at which the OD_{405} was 0.2. ND, not determined.

taining GPΔTM (0.5 μg/dose); cell-free, wild-type baculovirus-infected supernatants; gradient-purified MBGV (strain Musoke) (100 μg/dose); and gradient-purified MBGV (strain Ravn) (100 μg/dose). To evaluate protective immunity, each group was divided and half the animals challenged with guinea pig adapted MBGV (strain Musoke) and half with guinea pig adapted MBGV (strain Ravn). Results are shown in Table 3. ELISA titers were determined for each animal against both MBGV (strain Musoke) and MBGV (strain Ravn) antigens, using serum collected 2 days before challenge and 28 days after challenge. In groups 1 and 2 (GPΔTM), ELISA titers to MBGV (strain Musoke) were about fivefold higher than to Ravn antigen before challenge. Upon challenge with MBG-Mus, 4/5 animals in group 1 survived, with 1 animal succumbing on day 12, not significantly later than the mean day of death of control animals in group 3. No viremias were detected on day 7 in any of the survivors of group 1, whereas the animal that died had a detectable but low day 7 viremia. This contrasts with control animals in group 3 which all had high serum viremias (even the animal that survived demonstrated a 2.5 $\log_{10}$ viremia on day 7 postinfection). It was evident that all animals were exposed to virus, and that some viral replication had probably occurred, since a boost in serum antibody titer was observed in all surviving animals after challenge. Animals in group 2 that were challenged with MBGV (strain Ravn) all succumbed to infection with a mean day of death of 9 days. All animals demonstrated a high serum viremia on day 7 postinfection, with values no different than those of the control group 4 ($P = 0.94$).

Those animals in groups 5 and 6, which were immunized with MBGV (strain Musoke), and those in groups 7 and 8, which were immunized with MBGV (strain Ravn), all had high serum ELISA titers ($>10^5$) to both strains of MBGV prior to challenge. In addition, all animals challenged in these groups survived without regard to challenge virus. No viremia was detected in any of these animals. No boost in ELISA titer was observed in survivors, suggesting the possibility of sterile immunity.

Comparison of protection in strain 13 and Hartley guinea pigs using recombinant antigens from two different insect cell lines

Six groups of eight animals were immunized as described under Materials and Methods and Table 4. Three of those groups were made up of strain 13 guinea pigs and three were Hartley guinea pigs. Cell-free *Sf*9 supernatants containing GPΔTM or cell-free *Trichoplusia ni* supernatants containing GPΔTM were each administered to one group of Hartley guinea pigs and one group of strain 13 guinea pigs. The dose for each of these antigens was determined to be ~5 and 25 μg/injection, respectively. As controls, adjuvant in PBS was administered to the remaining group of Hartley guinea pigs, while

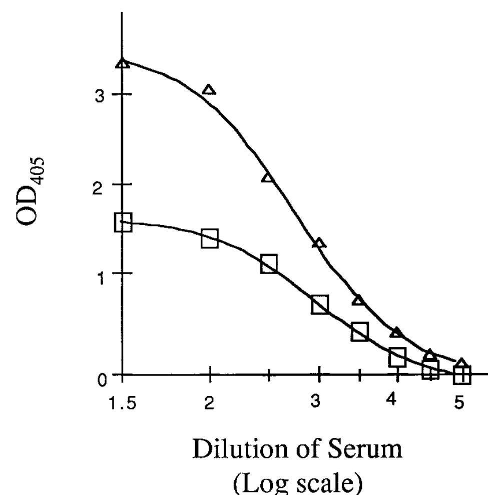

FIG. 2. Comparison of GP-specific versus anti-MBGV antibody responses in convalescent guinea pig serum. Graph of OD versus dilution of convalescent guinea pig serum assayed on either purified MBGV (strain Musoke) (△) or GPΔTM antigen (□).

TABLE 3

Results of MBGV Challenge of Strain 13 Guinea Pigs Vaccinated with Either Killed MBGV or Sf9-Produced GPΔTM

Group	Antigen	Challenge Day 0	Log_{10} GMT ELISA Titer ± SD: Musoke antigen Day −2	Musoke antigen Day 28	Ravn antigen Day −2	Ravn antigen Day 28	Log_{10} PFU/ml viremia GMT ± SD Day 7	MDD ± SD	S/T
1	GPΔTM	MBGV strain Musoke	4.2 ± 0.3	5.6 ± 0.3	3.5 ± 0.5	4.5 ± 0.1	2.7†	12†	4/5
2	GPΔTM	MBGV strain Ravn	4.0 ± 1.4	—	3.3 ± 1.1	—	4.8 ± 0.8	9 ± 1.6	0/5
3	Control baculovirus	MBGV strain Musoke	<1.5	—	<1.5	—	4.0 ± 1.0	11 ± 0.8	1/5
4	Control baculovirus	MBGV strain Ravn	<1.5	—	<1.5	—	4.7 ± 0.5	11 ± 0.0	0/5
5	Musoke virion	MBGV strain Musoke	5.6 ± 0.3	5.4 ± 0.3	5.1 ± 0.4	4.7 ± 0.4	<1.7	—	5/5
6	Musoke virion	MBGV strain Ravn	5.6 ± 0.2	5.5 ± 0.1	5.3 ± 0.3	5.0 ± 0.2	<1.7	—	5/5
7	Ravn virion	MBGV strain Musoke	5.0 ± 0.6	5.2 ± 0.6	5.3 ± 0.6	5.1 ± 0.4	<1.7	—	5/5
8	Ravn virion	MBGV strain Ravn	5.4 ± 0.2	5.8 ± 0.5	5.6 ± 0.3	5.4 ± 0.2	<1.7	—	5/5

Note. MDD, Mean day of death; S/T, survivors/total; GMT, geometric mean titer. †Represents data from the animal(s) that died. All geometric mean ELISA titers were determined using sera from surviving animals.

PBS was administered to the remaining group of Strain 13 guinea pigs. Before the time of challenge (day 0), the animals were divided into 12 groups, numbered 9–20, and challenged with either guinea pig-adapted, plaque-picked MBGV (strain Musoke), or guinea pig-adapted, plaque-picked MBGV (strain Ravn). ELISA titers were determined for each animal against both MBGV (strain Musoke) and MBGV (strain Ravn) antigens using serum collected 3 days prior to challenge and 33 days after challenge. No significant difference in response to antigen produced in Sf9 versus that produced in *Trichoplusia ni* was observed in either the Hartley or strain 13 guinea pigs ($P = 0.43$ and $P = 0.74$, respectively). In addition, the antibody responses as measured by ELISA were indistinguishable between strain 13 and Hartley guinea pigs.

Again, animals that received GPΔTM antigen followed by challenge with the MBGV (strain Musoke) had a higher survival rate than those challenged with MBGV (strain Ravn). In the Hartley groups that were immunized with GPΔTM and challenged with MBGV (strain Musoke) (groups 9 and 11), 4/5 animals survived in each group. Interestingly, the animal that died in group 9 did not have a detectable viremia on day 7, but time to death was not substantially delayed. In contrast the animal in group 11 that died had a high viremia on day 7. Animals in group 11 demonstrated a rise in antibody titer to both homologous and heterologous antigen when pre- and postchallenge sera were compared, while those in group 9 did not. The strain 13 guinea pigs immunized with GPΔTM and challenged with MBGV (strain Musoke) all survived challenge (groups 15 and 17). None of the animals in either of the groups had a detectable viremia on day 7, and no rise in antibody titer was observed after challenge in either group. In general, animals that were immunized with GPΔTM and infected with MBGV (strain Ravn) fared worse (50% survival) than those challenged with MBGV (strain Musoke) (90% survival).

Passive transfer of serum from immune animals into naive animals

To determine whether humoral immunity could be sufficient to protect guinea pigs from lethal MBGV challenge, a transfer of serum obtained from immune animals into naive animals, followed by challenge, was performed. Results are shown in Table 5. Three different immune sera were used for transfer. Group 1 was given convalescent pooled sera obtained 28 days after infection of strain 13 guinea pigs that had been infected with a guinea pig avirulent plaque-purified derivative of MBGV (strain Musoke). Group 2 animals were given a serum pool from strain 13 guinea pigs that had been immunized three times with MBGV (strain Musoke) virion and subsequently survived challenge with MBGV (strain Musoke) (group 5, day 28 serum, Table 3). Animals in groups 4 through 7 received dilutions of a serum pool from strain 13 guinea pigs that had been immunized three times with MBGV (strain Ravn) and subsequently survived challenge with MBGV (strain Ravn) (group 8, day 28 serum, Table

TABLE 4

Results of MBGV Challenge of Strain 13 and Hartley Guinea Pigs Vaccinated with Either Sf9 or *Tricoplusia ni*-Produced GPΔTM

Group[a]	Antigen	Challenge Day 0	Log_{10}GMT ELISA Titer ± SD: Musoke antigen Day −3	Musoke antigen Day 33	Ravn antigen Day -3	Ravn antigen Day 33	Log_{10} PFU/ml viremia GMT ± SD	MDD ± SD	S/T
9 (H)	Tni GPΔTM	MBGV strain Musoke	4.4 ± 0.3	4.1 ± 0.3	3.5 ± 0.3	3.3 ± 0.3	<1.7	12†	4/5
10 (H)	Tni GPΔTM	MBGV strain Ravn	3.9 ± 0.2	5.0 ± 0.3	2.7 ± 0.3	4.8 ± 0.1	5.3†	8†	2/3
11 (H)	Sf9 GPΔTM	MBGV strain Musoke	4.1 ± 0.2	5.2 ± 0.6	3.3 ± 0.3	4.4 ± 0.4	4.5†	9†	4/5
12 (H)	Sf9 GPΔTM	MBGV strain Ravn	4.1 ± 0.1	—	3.5 ± 0.5	—	4.2 ± 1.6	10 ± 3.0	0/3
13 (H)	Adjuvant	MBGV strain Musoke	<2.0	—	<2.0	—	5.9 ± 0.5	9 ± 1.0	0/5
14 (H)	Adjuvant	MBGV strain Ravn	<2.0	—	<2.0	—	5.4 ± 0.9	9 ± 1.0	0/3
15 (13)	Tni GPΔTM	MBGV strain Musoke	4.8 ± 0.2	4.2 ± 0.1	3.5 ± 0.1	2.9 ± 0.2	<1.7	—	5/5
16 (13)	Tni GPΔTM	MBGV strain Ravn	4.6 ± 0.4	5.0 ± 0.0	3.2 ± 0.2	4.8 ± 0.5	6.2†	8†	2/3
17 (13)	Sf9 GPΔTM	MBGV strain Musoke	4.6 ± 0.2	4.3 ± 0.5	3.2 ± 0.2	2.9 ± 0.2	<1.7	—	5/5
18 (13)	Sf9 GPΔTM	MBGV strain Ravn	4.8 ± 0.2	5.0 ± 0.4	3.3 ± 0.3	4.0 ± 1.8	<1.7	18†	2/3
19 (13)	PBS	MBGV strain Musoke	<2.0	—	<2.0	—	5.3 ± 0.2	11 ± 2.0	0/3
20 (13)	PBS	MBGV strain Ravn	<2.0	—	<2.0	—	5.6 ± 0.3	10 ± 1.0	0/5

Note. Tni, *Trichoplusia ni*-produced antigen. †Represents data from the animal(s) that died.
[a] (H) Represents groups of Hartley guinea pigs; (13) represents groups of Strain 13 guinea pigs.

3). Animals in groups 3 and 8 were given HBSS as a control. All animals in groups 1 and 2 survived challenge, indicating no substantial difference in the quality of antibody, whether evoked by sublethal infection or by killed virion in adjuvant. Not surprisingly, the same results were obtained when serum from MBGV (strain Ravn) immune animals was transferred to naive animals, followed by challenge with MBGV (strain Ravn) (group 4). Decreasing the amount of immune serum resulted in a decrease in efficacy: animals that received either 3 or 1 ml of serum

TABLE 5

Passive Transfer of Either Immune or Convalescent Guinea Pig Sera Can Protect Naive Guinea Pigs From Lethal Challenge with MBGV

Group	Gpig antiserum	Volume[a] (ml/Gpig)	Challenge (day 0)	Log_{10} PFU/ ml GMT ± SD Viremia (day 10)	S/T	MDD ± SD
1	Mus Convalescent	3.0	MBGV strain Musoke	<1.7	5/5	—
2	Mus Virion Immunized	3.0	MBGV strain Musoke	<1.7	4/4	—
3	HBSS control	3.0	MBGV strain Musoke	4.8 ± 0.2	1/5	11.5 ± 0.6
4	Ravn Virion Immunized	3.0	MBGV strain Ravn	<1.7	3/3	—
5	Ravn Virion Immunized	1.0	MBGV strain Ravn	<1.7	3/3	—
6	Ravn Virion Immunized	0.3	MBGV strain Ravn	4.6†	1/3	13 ± 0.0
7	Ravn Virion Immunized	0.1	MBGV strain Ravn	3.9 ± 0.6	0/3	13 ± 0.0
8	HBSS control	3.0	MBGV strain Ravn	—	0/3	9.3 ± 0.6

Note. All samples were adjusted to 3.0 ml total volume with sterile saline to control for any volume effects. †Represents data from the animal(s) that died.
[a] Volume represents the amount of serum administered to each animal.

were completely protected, with no viremias detected on day 10 (groups 4 and 5). Thus, any transferred serum amount of about 3.0 ml/kg (as calculated for group 5) or greater was fully effective. Of guinea pigs that received 0.3 ml immune serum, 1/3 survived (group 6). The two guinea pigs that died had high viremias on day 10, but died 3–4 days later than control animals. The guinea pig that survived had no detectable viremia on day 10. Suggesting a rather precise therapeutic cutoff in this experiment, the surviving animal in group 6 was smaller than the other two and thus received 1.5 ml serum/kg, as compared to the unprotected animals which received about 0.5 ml/kg. Animals that received only 0.1 ml of antiserum all succumbed to infection with high viremia on day 10, but with a time of death delayed by 3–4 days compared to control animals. Within the limits of this study, we did not observe any adverse effects of antibodies, such as increased viremias or accelerated disease.

DISCUSSION

To determine some of the requirements of a safe and effective vaccine against MBGV, we initiated studies to identify protective antigens present in the virion. The glycoprotein gene of MBGV (strain Musoke) was successfully cloned into a recombinant baculovirus vector and expressed in insect cells. In addition to GP, a truncated, secreted form of MBGV GP was produced. The GP and GPΔTM products were specifically recognized by anti-MBGV sera when immunoprecipitated from infected insect cell lysate, suggesting these products retained at least some of the authentic epitopes present in virion proteins. The faster migration of recombinant GP compared to authentic GP in SDS–PAGE has been extensively evaluated elsewhere (Becker *et al.,* 1996) and was most likely due to differential glycosylation in insect and mammalian cells. Interestingly, the recombinant GPΔTM product in supernatant has an apparent molecular mass that is less than virion GP but greater than either the full-length recombinant GP product or GPΔTM from cell lysates.

Characterization of the expressed proteins by lectin blots and ELISA demonstrated that the recombinant proteins differed in carbohydrate content compared to virion-associated GP, but retained their antigenicity with respect to several GP-specific Mabs. The glycosylation patterns of the *Sf*9-produced proteins was not substantially different from those reported by Becker *et al.* (1996). Strong reactivity with GNA and weak reactivity with DSA indicated that the product immunoprecipitated from GP-producing *Sf*9 cell lysates was composed of two forms of the protein. The majority of the protein contained unprocessed, high mannose-type glycans, while a minority of the protein contained processed, complex N-linked glycans. In addition, the reactivity with PNA indicates that at least of some of the protein was O-glycosylated and thus presumably trafficed from the endoplasmic reticulum through the Golgi network. Surprisingly a weak reaction with MAA was also observed, indicating the presence of small amounts of sialic acid residues on the protein. Although insect cells are thought either not to add sialic acid to glycoproteins or to trim off added sialic acid residues (Kretzchmar *et al.,* 1994; Jarvis and Finn, 1995), there are some reports indicating that insect cells may indeed add sialic acid to some proteins (Davis and Wood, 1995). Although the reaction of GP with MAA was weak, it was reproducible. In interpreting the importance of differences in glycosylation between *Sf*9 and Vero cells, it should be noted that differences in GP lectin-binding patterns were also observed when MBGV was grown in a variety of mammalian cell types (Feldmann *et al.,* 1994).

Further characterization of the recombinant proteins using Mabs directed against MBGV (strain Musoke) GP indicated that the GPΔTM was secreted into the serum-free supernatant of recombinant baculovirus-infected cells and contained several epitopes found in virion-associated GP. Sixteen of 17 MBGV GP-specific Mabs examined reacted equally well with GPΔTM and MBGV (strain Musoke). The difference in reactivity of III-5B8 with MBGV (strain Musoke) and GPΔTM may be due to an altered epitope or different glycosylation in GPΔTM that resulted in less efficient binding of this Mab. In addition, only two of the MBGV (strain Musoke) GP-specific Mabs examined reacted well with MBGV (strain Ravn) GP. The lack of reactivity of MBGV (strain Musoke) anti-GP Mabs with MBGV (strain Ravn) was most likely due to the heterogeneity of GP in these two (strains of MBGV) and explains the incomplete cross-protection observed in animals immunized with GPΔTM and challenged with MBGV-strain Ravn. Because the recombinant antigen reacted so well in ELISA with Mabs, and the relative ease with which large quantities can be made, there is potential for use of this antigen as a diagnostic reagent. To investigate this possibility further, the GPΔTM antigen was compared to purified MBGV as an ELISA antigen on a titration of convalescent guinea pig serum. The results demonstrated that the GPΔTM was a useful tool in distinguishing anti-GP response from responses to other virion proteins.

Using the guinea pig model, the efficacy of both a prototypical killed virus vaccine and the recombinant GPΔTM protein were examined. Gradient-purified, irradiated virus was able to completely protect strain 13 guinea pigs from challenge with either MBGV (strain Musoke) or MBGV (strain Ravn). While protection of the recombinant GPΔTM-immunized animals was achieved against MBGV (strain Musoke), protection was more difficult to achieve in animals challenged with MBGV (strain Ravn). This lack of cross-protection may have been due to either a difference in the quantity or quality of antibody produced by animals immunized with GPΔTM compared to those immunized

with virion. Since GPΔTM was cloned from MBGV (strain Musoke) and the MBGV (strain Musoke) and MBGV (strain Ravn) GP proteins differ by 22% in amino acid sequence, the deficiency of cross-protection observed was not wholly unexpected. Animals immunized with GPΔTM may contain antibodies that neutralize MBGV (strain Musoke) well, but not MBGV (strain Ravn). Attempts were made to measure neutralizing antibodies in the guinea pig sera using a plaque-reduction neutralization test to address this question; however, titers were low and no correlation between protection and presence of neutralizing antibody was observed. Because of the difficulty in quantitating MBGV neutralizing antibody levels in serum, the absence of correlation observed may be the result of insufficient sensitivity of this assay for filoviruses. In addition, the virion-immunized animals developed antibodies not only against GP but also against the other major virion proteins, while the GPΔTM-immunized animals only produced antibodies directed against GP. Since the GP protein is expected to be the most variable of the virion proteins, it remains possible that immune responses to other virion proteins, which are more conserved, may result in more efficient cross-protection. A third possibility is that the amount of antibody produced in GPΔTM-immunized animals was insufficient for cross-protection. This dose effect may be responsible for the observation in the second protection experiment where strain 13 and Hartley guinea pigs were immunized with higher doses of GPΔTM compared to the first experiment and an observed increase in the protection of animals challenged with MBGV (strain Ravn) was observed. Furthermore, since the determinants of cross-protection are unknown, it remains possible that epitopes responsible for cross-protection are missing or altered in GPΔTM. Finally, a qualitative difference between the immune response to virion GP and GPΔTM might result in a more cross-protective immune response in the guinea pigs that received virion as an immunogen.

To determine whether serum antibodies mediated protection, a serum transfer study was performed. The results clearly indicated that serum from either immunized or convalescent animals could confer protection to naive guinea pigs challenged with lethal doses of MBGV. Furthermore, the protection was dependent on the amount of serum administered. While the result of this experiment demonstrates that protection from a homologous strain of MBGV can be conferred by antibody alone, the role of cell-mediated immunity in protection from homologous, and more importantly heterologous, subtypes of MBGV cannot be dismissed.

In other studies not presented here, we also examined the immunogenicity of baculovirus-expressed NP- and VP-40. Insect cell lysates containing high levels of NP- and VP-40 in the presence of adjuvant were used to inoculate guinea pigs. Protection was negligible, as none (0/5) of the animals in either group survived challenge (data not shown). However, the role of NP- and/or VP-40 in protection cannot be excluded, as the presentation of the antigen may play a key role in developing effective immunity. Specifically, the induction of a cytotoxic T-cell response by these antigens may be required for effective protection. Further studies of the protective efficacies of NP- and VP-40 using methods of immunization that favor a T-cell response may prove more fruitful.

Historically, the prospects for an effective vaccine against filovirus infection was questionable at best. Here we were able to demonstrate, in a guinea pig model system, that the glycoprotein gene product can serve as a protective immunogen for MBGV. While antigenic variation of MBGV makes a monovalent vaccine problematic, complete protection against two types of MBGV was obtained using killed virus as an immunogen. Further work is required to determine what the determinants of cross-protection are. These may include antibody to other viral proteins or cell-mediated immunity directed against one or more of the viral proteins. In addition, the effectiveness of a subunit vaccine in nonhuman primates must be evaluated. Results in nonhuman primates have shown that passive protection from Ebola infection is easier to achieve in guinea pigs than in primates (Jahrling *et al.,* 1996). Thus, the promise of any vaccination strategy for filoviruses should be viewed with caution until protection is also demonstrated in nonhuman primates.

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