DNA Vaccines Expressing either the GP or NP Genes of Ebola Virus Protect Mice from Lethal Challenge

Lorna Vanderzanden,* Mike Bray,* Deborah Fuller,† Tim Roberts,‡ David Custer,* Kristin Spik,* Peter Jahrling,* John Huggins,* Alan Schmaljohn,* and Connie Schmaljohn†,1

*Virology Division, United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Maryland 21702-5011; and †PowderJect Vaccines, Inc., Madison, Wisconsin 53711

Received January 20, 1998; returned to author for revision February 23, 1998; accepted April 3, 1998

INTRODUCTION

Ebola virus (EBOV), a member of the family Filoviridae, causes severe viral hemorrhagic fever and high mortality in humans. The ecology and epidemiology of EBOV are poorly understood and the host species that maintains the virus in nature is unknown. Although aerosol transmission of EBOV was demonstrated in primates (Jaax et al., 1995), most nosocomial or human-to-human transmission has been correlated with direct contact with infected patients or their bodily fluids (Peters et al., 1994; Feldmann and Klenk, 1996). To date, three distinct EBOV strains have been found to be pathogenic for humans: Zaire, Sudan, and Ivory Coast (Peters et al., 1994; Le Guenno et al., 1995). A fourth strain, Reston, appears to be nonpathogenic for humans but causes hemorrhagic fever in nonhuman primates (Jahrling et al., 1990).

EBOV caused two major outbreaks of Ebola hemorrhagic fever (EHF), in 1976 and 1995, during which hundreds of cases were reported, with mortality rates of approximately 80–90% (CDC, 1995a,b; Peters et al., 1994). EBOV isolates from these outbreaks were antigenically and genetically very similar; thus the same virus was responsible for both epidemics (Sanchez et al., 1996).

EBOV particles consist of helical nucleocapsids containing approximately 19 kb of single-strand, negative-sense RNA. The ribonucleocapsids are surrounded by a membrane of host origin from which a highly glycosylated type 1 transmembrane protein (GP) protrudes (Feldmann et al., 1994; Buchmeier et al., 1983; Sanchez et al., 1993; Kiley et al., 1980, 1988; Geisbert and Jahrling, 1990). The EBOV genome encodes seven proteins with a gene order of NP-VP35-VP40-GP-VP30-VP24-L (Elliott et al., 1993; Sanchez et al., 1993). Each product is produced from a separate, polyadenylated mRNA. GP is encoded in two adjoining reading frames which are connected by transcriptional editing. The editing involves inserting a nontemplated adenosine into a stretch of seven adenosines in the mRNA (Sanchez et al., 1996). In addition to GP, VP 24, a minor envelope protein, is associated with virions. The helical ribonucleocapsids contain four of the viral proteins: NP, the major nucleocapsid protein; L, an RNA-dependent RNA polymerase; VP30; and VP35. The roles of these latter two proteins are not clearly defined, but by analogy to Marburg virus (MBGV) and to viruses in the Paramyxoviridae family, they may be involved in regulation of gene expression or replication. VP40 is the matrix protein, which permits interaction between membrane components and NP (Sanchez et al., 1993).

Currently, no effective vaccines or therapies for EHF have been reported. Conventional approaches to vaccine development, such as inactivation or attenuation of virulent viruses, pose risks to researchers, manufacturers, and recipients of such vaccines. Recombinant DNA approaches offer means to eliminate the risk associated with production of the vaccines as well as the risk of incomplete inactivation or reversion to wild-type virus. The naked DNA approach has proven to be a simple and...
effective means for generating protective immunity to a variety of pathogens in a number of animal models (e.g., Fuller and Haynes, 1994; Fynan et al., 1993, 1995; Pertmer et al., 1995; Schmaljohn et al., 1997; Ulmer et al., 1995, 1996a,b; Whalen, 1996). One major advantage of this approach, compared to the use of inactivated or preformed subunit vaccines, is that the foreign genes are transcribed within the host's own cells; thus, they mimic natural infection, resulting in both cell-mediated and humoral immune responses (Eisenbraun et al., 1993; Fynan et al., 1993; Haynes et al., 1994; Pertmer et al., 1995). Gene gun immunization permits the use of very small amounts of DNA because the DNA is delivered directly into host cells rather than into intercellular spaces. Moreover, the site of gene gun inoculation, the skin, is an immunologically active tissue (Stingl, 1993), and the potential for direct delivery of DNA into antigen presenting cells (i.e., dendritic cells) is present (Condon et al., 1996).

Although immune mechanisms for preventing and clearing filovirus infections are not well understood, there appears to be some association between the presence of neutralizing antibody and protection. Passive transfer of hyperimmune guinea pig sera was demonstrated to protect guinea pigs from challenge with MBGV (Hevey et al., 1997). Similarly, passive transfer of equine IgG to EBOV protected guinea pigs (Jahrling et al., 1996) and baboons (Mikhailov et al., 1994) from a subsequent EBOV challenge. However, passive transfer of the same sera to cynomolgus monkeys did not afford protection (Jahrling et al., 1996). It was suggested that because cynomolgus monkeys develop higher viremias than baboons, it was not possible to overcome the viral load with the transferred antibody (Jahrling et al., 1996). Thus, elicitation of neutralizing antibody to EBOV appears to be a desirable objective for a vaccine, but it may not be sufficient for protection and may not even be the primary means of protection.

EBOV GP is the most likely viral protein to elicit neutralizing antibodies, because it is the only protein known to be on the virion surface. Also, monoclonal antibodies to GP of MBGV were found to neutralize infectious virus in cell culture assays (A. Schmaljohn, unpublished data). GP is also a reasonable candidate for a subunit vaccine, as demonstrated with baculovirus-expressed MBGV GP (Hevey et al., 1997) and vaccinia virus-expressed EBOV GP (Gilligan et al., 1997), both of which were able to elicit partially protective immune responses in guinea pigs. If a strong cell-mediated immune response is also needed for effective protection from filovirus infections, then other proteins such as NP may also prove useful.

In the studies reported here, we examined the potential of the GP and NP genes of EBOV to elicit protective immunity. An adult mouse model recently developed and described (Bray et al., 1996, 1998) was used in lieu of guinea pigs in order to more easily achieve significant group sizes in a restrictive BSL4 environment and to facilitate analyses of cellular immune mechanisms.

RESULTS

Cloning and sequence analysis of the EBOV GP and NP genes

Two different EBOV, strain Zaire 95, GP genes were generated by RT±PCR. One of the cDNAs encompassed only the coding region of the GP gene and the other included an additional 139 nucleotides of 5’ noncoding information. The latter construct was produced so that we could determine if the noncoding sequences, which form a potential stem-loop structure (Sanchez et al., 1993), might influence expression of GP in our system. Both cDNAs were cloned into the plasmid vector pCRII and later were subcloned into the plasmid vector pWRG7077. Our GP gene sequences differed from that reported previously for EBOV, strain Zaire 1995 (GenBank Accession No. U28077), at only 5 nucleotides. Four of the nucleotide differences resulted in substitutions of predicted amino acids (E → D at position 93; S → T at position 355; G → A at position 415; and F → I at position 601 with respect to the methionine initiation codon for EBOV GP). As reported previously (Sanchez et al., 1993, 1996; Volchkov et al., 1995), the complete GP of EBOV is encoded in a reading frame that is generated by transcriptional editing. The editing involves inserting a non-templated adenosine into a stretch of seven other templated adenosines. By sequencing numerous clones, we found that there was a great variation in the number of A residues at the transcriptional editing site, with as few as 4 A residues to as many as >50 residues. We did not determine if this observation was related to reverse transcription or PCR artifacts or if in fact the viral RNA population varied this extensively. We selected two of the clones with the correct number of A residues (8) for further analysis of GP expression.

The NP gene of EBOV was produced by RT±PCR, cloned into the plasmid pCRII, and then subcloned into pWRG7077. The gene was sequenced and compared to that previously reported for a 1976 isolate of EBOV (Sanchez et al., 1993; GenBank Accession No. L11365). The sequences differed by 35 of 2220 nucleotides and in 8 of 739 predicted amino acids. We deposited the NP nucleotide sequence from this 1995 EBOV isolate in GenBank as AF054908.

Transient expression of the GP and NP genes

Transient expression was assayed for the two EBOV GP genes and the NP gene which were cloned into pCRII and pWRG7077 vectors. The expression assay for the pCRII constructs employed a recombinant vaccinia virus expressing T7 polymerase for transcription of the gene. The expression assay for the pWRG7077 constructs re-
markers (not shown) are indicated to the left of each autoradiograph. The positions of radiolabeled protein products in both assay systems (Fig. 1). These findings indicate that although the noncoding nucleotides (stem-loop structures) found at the 5′ ends of the authentic EBOV GP mRNAs may have some role in viral replication, they are unimportant for in vitro expression, at least in the two systems that we tested. Transient expression of the EBOV NP yielded an expression product of the predicted size (Fig. 1).

Immunogenicity of the GP naked DNA vaccine

Our initial studies focused on evaluation of GP as a candidate vaccine because it is the only major surface protein of virions and was therefore the most logical candidate for eliciting neutralizing antibodies. To determine if the GP DNA was able to elicit protective immunity in mice, we vaccinated groups of 10 BALB/c mice by delivering DNA-coated gold beads to the abdominal epidermis by particle bombardment with helium pressure using the Powderject-XR gene gun (Powderject Vaccines, Inc. Madison, WI). The first four groups tested received one, two, three, or four vaccinations of 0.5 μg of EBOV DNA or control DNA at 4-week intervals. Mice were challenged with mouse-adapted EBOV 12 weeks after the single inoculation, 8 weeks after the second of two inoculations, or 4 weeks after the third or fourth inoculations. A clear dose response of protection was observed with increasing numbers of vaccinations, and a delay in time to death was observed for some of the vaccinated mice that succumbed to the challenge (Fig. 2). Increasing the number of vaccinations to five with 0.5 μg of DNA did not improve the survival of mice after challenge.

Because only partial protection was achieved with the GP doses and regimens tested, we inoculated additional groups of mice with a priming dose of 0.5 μg of DNA and increased the dosage of DNA in the second, third, and fourth boosts to 1.5 μg of DNA. The boosts were given at 4-week intervals and the mice were challenged approximately 8 weeks after the third boost or 4 weeks after the fourth boost. All mice survived the challenge (Fig. 2).

Survival of mice in each group (Fig. 2) that received two or more EBOV DNA inoculations was significantly better than that of control mice (log rank analysis, P values: two doses 0.0042; three doses 0.0002; four doses 0.0001; five doses <0.0001; either group receiving 1.5 μg, <0.0001). Survival of mice whose regimen included doses of 1.5 μg of DNA (Fig. 2) was significantly better than that of mice that received one, two, three, or five doses of 0.5 μg of DNA (log rank analysis, P values: one dose <0.0001; two doses 0.0041; three doses 0.0289; five doses 0.0117) but not significantly better than that of mice which received four doses of 0.5 μg of DNA (P = 1.246).

To assess the ability of the vaccine to reduce viremia, a factor that we have found to be predictive of subsequent survival, we administered 0.5 μg of EBOV GP DNA inoculations. A clear dose response of protection was observed with increasing numbers of vaccinations, and a delay in time to death was observed for some of the vaccinated mice that succumbed to the challenge (Fig. 2). Increasing the number of vaccinations to five with 0.5 μg of DNA did not improve the survival of mice after challenge.

Because only partial protection was achieved with the GP doses and regimens tested, we inoculated additional groups of mice with a priming dose of 0.5 μg of DNA and increased the dosage of DNA in the second, third, and fourth boosts to 1.5 μg of DNA. The boosts were given at 4-week intervals and the mice were challenged approximately 8 weeks after the third boost or 4 weeks after the fourth boost. All mice survived the challenge (Fig. 2).

Survival of mice in each group (Fig. 2) that received two or more EBOV DNA inoculations was significantly better than that of control mice (log rank analysis, P values: two doses 0.0042; three doses 0.0002; four doses 0.0001; five doses <0.0001; either group receiving 1.5 μg, <0.0001). Survival of mice whose regimen included doses of 1.5 μg of DNA (Fig. 2) was significantly better than that of mice that received one, two, three, or five doses of 0.5 μg of DNA (log rank analysis, P values: one dose <0.0001; two doses 0.0041; three doses 0.0289; five doses 0.0117) but not significantly better than that of mice which received four doses of 0.5 μg of DNA (P = 1.246).

To assess the ability of the vaccine to reduce viremia, a factor that we have found to be predictive of subsequent survival, we administered 0.5 μg of EBOV GP DNA inoculations. A clear dose response of protection was observed with increasing numbers of vaccinations, and a delay in time to death was observed for some of the vaccinated mice that succumbed to the challenge (Fig. 2). Increasing the number of vaccinations to five with 0.5 μg of DNA did not improve the survival of mice after challenge.

Because only partial protection was achieved with the GP doses and regimens tested, we inoculated additional groups of mice with a priming dose of 0.5 μg of DNA and increased the dosage of DNA in the second, third, and fourth boosts to 1.5 μg of DNA. The boosts were given at 4-week intervals and the mice were challenged approximately 8 weeks after the third boost or 4 weeks after the fourth boost. All mice survived the challenge (Fig. 2).

Survival of mice in each group (Fig. 2) that received two or more EBOV DNA inoculations was significantly better than that of control mice (log rank analysis, P values: two doses 0.0042; three doses 0.0002; four doses 0.0001; five doses <0.0001; either group receiving 1.5 μg, <0.0001). Survival of mice whose regimen included doses of 1.5 μg of DNA (Fig. 2) was significantly better than that of mice that received one, two, three, or five doses of 0.5 μg of DNA (log rank analysis, P values: one dose <0.0001; two doses 0.0041; three doses 0.0289; five doses 0.0117) but not significantly better than that of mice which received four doses of 0.5 μg of DNA (P = 1.246).

To assess the ability of the vaccine to reduce viremia, a factor that we have found to be predictive of subsequent survival, we administered 0.5 μg of EBOV GP DNA inoculations. A clear dose response of protection was observed with increasing numbers of vaccinations, and a delay in time to death was observed for some of the vaccinated mice that succumbed to the challenge (Fig. 2). Increasing the number of vaccinations to five with 0.5 μg of DNA did not improve the survival of mice after challenge.

Because only partial protection was achieved with the GP doses and regimens tested, we inoculated additional groups of mice with a priming dose of 0.5 μg of DNA and increased the dosage of DNA in the second, third, and fourth boosts to 1.5 μg of DNA. The boosts were given at 4-week intervals and the mice were challenged approximately 8 weeks after the third boost or 4 weeks after the fourth boost. All mice survived the challenge (Fig. 2).

Survival of mice in each group (Fig. 2) that received two or more EBOV DNA inoculations was significantly better than that of control mice (log rank analysis, P values: two doses 0.0042; three doses 0.0002; four doses 0.0001; five doses <0.0001; either group receiving 1.5 μg, <0.0001). Survival of mice whose regimen included doses of 1.5 μg of DNA (Fig. 2) was significantly better than that of mice that received one, two, three, or five doses of 0.5 μg of DNA (log rank analysis, P values: one dose <0.0001; two doses 0.0041; three doses 0.0289; five doses 0.0117) but not significantly better than that of mice which received four doses of 0.5 μg of DNA (P = 1.246).

To assess the ability of the vaccine to reduce viremia, a factor that we have found to be predictive of subsequent survival, we administered 0.5 μg of EBOV GP DNA inoculations. A clear dose response of protection was observed with increasing numbers of vaccinations, and a delay in time to death was observed for some of the vaccinated mice that succumbed to the challenge (Fig. 2). Increasing the number of vaccinations to five with 0.5 μg of DNA did not improve the survival of mice after challenge.

Because only partial protection was achieved with the GP doses and regimens tested, we inoculated additional groups of mice with a priming dose of 0.5 μg of DNA and increased the dosage of DNA in the second, third, and fourth boosts to 1.5 μg of DNA. The boosts were given at 4-week intervals and the mice were challenged approximately 8 weeks after the third boost or 4 weeks after the fourth boost. All mice survived the challenge (Fig. 2).

Survival of mice in each group (Fig. 2) that received two or more EBOV DNA inoculations was significantly better than that of control mice (log rank analysis, P values: two doses 0.0042; three doses 0.0002; four doses 0.0001; five doses <0.0001; either group receiving 1.5 μg, <0.0001). Survival of mice whose regimen included doses of 1.5 μg of DNA (Fig. 2) was significantly better than that of mice that received one, two, three, or five doses of 0.5 μg of DNA (log rank analysis, P values: one dose <0.0001; two doses 0.0041; three doses 0.0289; five doses 0.0117) but not significantly better than that of mice which received four doses of 0.5 μg of DNA (P = 1.246).

To assess the ability of the vaccine to reduce viremia, a factor that we have found to be predictive of subsequent survival, we administered 0.5 μg of EBOV GP DNA inoculations. A clear dose response of protection was observed with increasing numbers of vaccinations, and a delay in time to death was observed for some of the vaccinated mice that succumbed to the challenge (Fig. 2). Increasing the number of vaccinations to five with 0.5 μg of DNA did not improve the survival of mice after challenge.
or control DNA three times to groups of 10 mice. The mice were challenged 4 weeks after the final vaccination and were terminally exsanguinated 3 or 5 days after challenge. Serum viremias were assessed by plaque assay. Three days after challenge, none of the 4 vaccinated mice examined were viremic, while the 4 control mice all had viremia titers greater than $10^5$ PFU/ml (Fig. 3). At 5 days after challenge, all 5 of the control mice had viremic titers $>10^8$ PFU/ml, while 2 of 6 vaccinated mice had titers $<10^3$ PFU/ml, and 1 mouse was still aviremic. The other 3 mice also had lower viremic titers than control mice (Fig. 3). We did not measure the replication of virus in organs or tissues other than blood because in our previous studies we found that levels of viremia correlated well with viral replication in the target organs, spleen and liver (Bray et al., 1998; Bray et al., unpublished information).

Three additional groups of mice that received the same vaccine regimen as those that were examined for viremia were challenged at 12, 24, or 36 weeks after the final vaccination to measure the duration of protective immunity elicited by the GP DNA vaccine. Seven of 10 mice survived the 12-week challenge, 2 of 9 survived the 24-week challenge, and 5 of 10 survived the 36-week challenge. Of the 40 control mice challenged at the four various time points of this study, mortality was 10/10, 10/10, 9/10, and 9/10. These data indicate that GP alone can afford complete protective immunity to EBOV in mice and that partial immunity lasts for at least 9 months.

In some studies with other DNA vaccines, increasing the time between vaccination improved immunogenicity of the vaccine (Fuller et al., 1997). To investigate whether a longer resting time between vaccinations might provide the same benefits as an additional vaccination, we inoculated groups of 20 mice with 0.5 μg of DNA either three times at 4-week intervals or two times at a 6-week interval. Four weeks after the final vaccination, the mice were challenged with EBOV. Although the prechallenge ELISA titers of mice that received three vaccinations were higher than those that received two vaccinations, there was no difference in the number of survivors (Fig. 4). Therefore, the longer resting period resulted in comparable protec-

FIG. 3. Viremia of EBOV DNA-inoculated or control mice challenged with mouse-adapted EBOV. Mice were vaccinated five times with 0.5 μg of EBOV GP DNA at 4-week intervals and then challenged with mouse-adapted EBOV 4 weeks after the final vaccination. Viremia was measured at days 3 and 5 postinfection (PI).

FIG. 4. ELISA titers of mice vaccinated with EBOV GP DNA. Mice were vaccinated two times at a 6-week interval (A) or three times at 4-week intervals (B) by gene gun inoculation of approximately 0.5 μg of DNA. Sera were analyzed by ELISA 4 weeks after the final vaccination at dilutions of $1.5 \pm 3.0 \log_{10}$. Titers of mice that survived challenge are shown to the left of the dotted lines.
tion with fewer immunizations. There was apparently no strong correlation of ELISA antibody titer and survival, as 2 of the mice with the highest antibody titers died, and 2 of the mice with the lowest antibody titers survived (Fig. 4).

Although we did not measure neutralizing antibody responses for all groups of mice, we did perform plaque reduction neutralization tests (PRNT_{50}) on pooled sera from mice that had received one, two, or three doses of 0.5 \( \mu \text{g} \) or 1.5 \( \mu \text{g} \) of DNA. For all groups of mice, prechallenge PRNT_{50} titers were <1:20 and postchallenge titers were 1:80. These data are consistent with other studies in which we found that only low levels of neutralizing antibodies were detected in either control or vaccinated mice that survive EBOV challenge and resultant disease (unpublished information).

To determine if the naked DNA, GP vaccine was also able to elicit cell-mediated immune responses in mice, 0.5 or 1.5 \( \mu \text{g} \) of the GP DNA vaccine was administered from one to five times at 4-week intervals, and cytotoxic T lymphocytes of individual mice were measured by chromium release assays after each dose on target cells expressing EBOV GP. Splenocytes from all seven mice tested that received at least two vaccinations with 1.5 \( \mu \text{g} \) of DNA demonstrated a range of maximum specific lysis of 9–40% (Fig. 5). Spleen cells from four of seven mice that received at least 2 vaccinations with 0.5 \( \mu \text{g} \) of DNA also specifically lysed the target cells (Fig. 5).
Comparing EBOV GP and NP candidate vaccines

Although GP alone offered protection from EBOV challenge, at least four vaccinations were required. For a human vaccine, it is desirable to elicit immunity with the lowest possible number of vaccinations. Also, based on the findings that passive transfer of EBOV immune horse serum offers only incomplete protection to some nonhuman primates (Jahrling et al., 1996) and on studies that showed only sporadic protection of guinea pigs with passively transferred neutralizing monoclonal antibodies to MBGV (A. Schmaljohn, unpublished information), we decided to test the vaccine potential of an additional EBOV gene, NP.

To compare the vaccine potential of NP and GP, we inoculated groups of 14 mice each with approximately 0.5 or 3 μg of each of the two candidate vaccines. The mice received three vaccinations at 4-week intervals, and their antibody titers to GP or NP were measured by ELISA 4 weeks after each vaccination. Antibodies were detectable after the first inoculation with either vaccine and rose after each of the two subsequent vaccinations (Fig. 6A). Ten mice from each group were challenged 4 weeks after the final vaccination. Partial protection was achieved with both GP and NP vaccines (Fig. 6B) and no statistically significant difference between the two vaccines was found (Breslow statistical analysis). As observed with GP inoculations, higher antibody titers detected by ELISA did not predict better protection.

A preliminary assessment of CTL responses was performed on pooled spleen cells from two mice from each of the NP groups and from the group that received 3 μg of GP DNA. The ELISA titers for the mice used in the chromium release assays were all >1:1000. Target cells for the NP-immunized mice were transformed P815 cells expressing NP. Specific lysis of NP and GP targets was observed (Fig. 7) and, for the small number of samples tested, appeared to be consistent with the GP data (Fig. 5) suggesting better CTL responses in mice that received higher amounts of DNA.

DISCUSSION

The filoviruses, MBGV and EBOV, are among the most deadly of known human pathogens. Control measures are impossible because the natural ecology, vector(s), and mode of transmission are largely unknown. Therefore, vaccination appears to be the only potential means

FIG. 6. Comparison of antibody responses and survival after challenge of mice vaccinated with the EBOV GP and NP candidate vaccines. (A) ELISA titers were determined for individual mice after each of three vaccinations with approximately 0.5 or 3 μg of NP or GP DNA, and geometric mean titers (GMT) were calculated. (B) The mice were challenged with 30 LD₅₀ of mouse-adapted EBOV. Results are shown for the first 15 days, but remained the same during the rest of the 21-day observation period.

FIG. 7. Comparison of CTL responses of mice vaccinated with NP or GP DNA. Two mice from each NP group and from the GP group receiving 3 μg of DNA (shown in Fig. 6A) were selected for CTL assays. Chromium release assays were performed as described under Materials and Methods and in the legend to Fig. 5. Targets were transformed P815 cells expressing EBOV GP or NP. The results shown are for pooled spleens of the two mice.
for protecting at-risk individuals from hemorrhagic fever caused by these viruses. Our studies were intended to determine if DNA vaccines can provide protection from EHF in an adult, immunocompetent mouse model. Use of a mouse model allowed us to measure protection by our DNA vaccines in statistically significant group sizes and will facilitate further evaluation of cell-mediated immune responses. However, it is not yet known whether either of the rodent models commonly used for filovirus studies (guinea pigs and mice) accurately mimic human disease; thus all such studies will require confirmation in nonhuman primates.

For our studies, we used a Zaire 1995 strain of EBOV to generate gene products. Sequence analysis of the GP and NP genes of this isolate confirmed earlier findings with the GP gene (Sanchez et al., 1996), which indicated that the virus which caused the 1995 EHF outbreak in Zaire was the same virus that caused an outbreak in that region in 1976. These findings suggest that EBOV is stably maintained in nature.

Although it is not completely clear what is needed to afford protective immunity to filovirus infections, it is likely that both humoral and cell-mediated immune responses will be required. Passive transfer of neutralizing antibodies can protect guinea pigs from filovirus infections (Jahrling et al., 1996; Hevey et al., 1997); thus, in some animal models, humoral immunity alone is sufficient for protection. However, because filoviruses replicate so quickly and to such high titers, it may not always be possible to overcome infection without the presence of a vigorous cell-mediated immune response in addition to an antibody response. Studies to define the filovirus antigens best suited for elicitation of protective immune responses are still in progress. Preliminary experiments with vaccinia virus recombinants demonstrated that protection from EBOV challenge could be obtained by immunizing guinea pigs with recombinants expressing GP but not with those expressing NP, VP35, VP40, or sGP (Gilligan et al., 1997). Similarly, protection from MBGV challenge could be obtained in guinea pigs immunized with baculovirus-expressed MBGV GP, but not NP (Hevey et al., 1997). In more recent studies, MBGV NP expressed from alphavirus recombinants conferred protective immunity to guinea pigs (A. Schmaljohn et al., unpublished information).

As we were preparing this paper, a study appeared in which DNA vaccines for EBOV, delivered by intramuscular injection, were assessed in mice and guinea pigs (Xu et al., 1998). In that study, nonneutralizing antibody responses could be elicited in mice by three intramuscular inoculations of 50 μg of plasmid DNA containing EBOV NP or GP cDNA. Only mice receiving the GP DNA had demonstrable CTL responses. Because a guinea pig-adapted strain of EBOV was used in that study, the mice were not challenged; thus protection could not be correlated with the observed CTL or antibody responses. In the same study, guinea pigs given four intramuscular injections of 100 μg of EBOV GP or NP cDNA at 2-week intervals were protected from challenge for 10 days when the challenge was performed 20 days after the final inoculation. However, when the vaccine was given two times at 2-week intervals, followed by a boost 4 weeks later and another boost 10 weeks after that, only the mice that received the GP DNA were protected from a challenge delivered 10 days after the final boost. Because the guinea pigs were killed 10 days after challenge for assay of viral antigen and virus in organs, it was not possible to determine if delayed deaths due to partial protection by the vaccine would have occurred at later times. Assessment of antibody responses by ELISA revealed that antibodies to both NP and GP were generated but that only GP-immunized guinea pigs were positive in cell proliferation and T cell growth factor assays. The authors concluded that cell-mediated but not humoral immune responses correlated with protection; however, that conclusion does not seem to be supported by their observation of protection of NP-immunized guinea pigs in the absence of detectable cell-mediated immune responses, nor by previous reports that passively transferred immune serum can protect guinea pigs from EBOV and MBGV infection (Jahrling et al., 1996; Hevey et al., 1997).

In our study, we were able to demonstrate significant and long-lived protection from EBOV challenge in mice immunized with very small quantities of the GP DNA. Consistent with earlier studies (Gilligan et al., 1997), neutralizing antibody responses to GP were low or undetectable before challenge and rose to titers of only 1:40 to 1:80 after challenge. We know that the mouse-adapted EBOV used for our challenge studies can be neutralized in such assays by antisera to nonadapted EBOV (Bray et al., 1998), so our finding is not an artifact of the mouse model. The finding is also consistent with other studies in our laboratory, including an attempt to produce hyperimmune mouse ascitic fluid to EBOV by priming with non-mouse-adapted EBOV in Freund’s adjuvant, followed by repeated large doses of a suckling mouse brain preparation of mouse-adapted virus in incomplete Freund’s adjuvant. The resultant ascitic fluid had a PRNT titer of only 1:40, suggesting that GP was only weakly immunogenic and that protection is not mediated by antibody alone. In this study, we demonstrated the induction of vigorous CTL responses specific for both NP and GP, suggesting a potential role for CTL for protection against EBOV in this mouse model.

In addition to protection by a GP DNA vaccine, we demonstrated that 3-μg quantities or less of NP DNA can also elicit protective immunity in mice. No statistically significant difference in protection between the GP and NP vaccines was observed. Thus, the gene gun method of DNA inoculation appears to be an efficient means to elicit protective immunity to EBOV in mice, and both the
GP and NP genes show promise as candidate vaccines capable of eliciting both cellular and humoral immune responses. Further studies are planned to investigate a combination GP/NP DNA vaccine, to evaluate the vaccines in nonhuman primates, and to examine in more detail the role of CTL in protection. Clearly, it is also essential that we further define important parameters for protective immunity to filoviruses so that we may, if necessary, redirect our vaccines toward elicitation of the most appropriate immune response.

MATERIALS AND METHODS

Viruses, cells, and medium

EBOV, strains Zaire 76 (also known as Mayinga strain) and Zaire 95, respectively, were originally isolated from patients during 1976 and 1995 outbreaks of Ebola hemorrhagic fever in Africa (CDC, 1995a,b; Johnson et al., 1977; Sanchez et al., 1996). To select a variant of EBOV that was pathogenic for mice, the EBOV (strain Zaire 76) was serial passaged in progressively older suckling BALB/c mice, and a ninth-passage isolate which was uniformly lethal for adult BALB/c mice was plaque-purified (Bray et al., 1996, 1998). This mouse-adapted EBOV was propagated in Vero E6 cells (ATCC C1008, CRL1586), aliquotted, and used in all mouse-challenge experiments. A stock of EBOV, strain Zaire 76, which had been passaged twice in Vero cells, was used in plaque-reduction neutralization assays. Transient expression assays were performed in baby hamster kidney (BHK) cells or COS cells. Cells were maintained in Eagle's minimal essential medium supplemented with 2% fetal bovine serum and antibiotics. Virus propagation was performed under biosafety level 4 (BSL-4) containment.

P815 mouse cells used for CTL assays were maintained in RPMI 1640 (Biowhittaker) containing 10% heat-inactivated FBS and antibiotics.

Mice

Female BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). Following DNA immunization and blood sampling, the animals were transferred to a BSL-4 facility for EBOV challenge. The mice were then observed on a daily basis for at least 21 days for signs of illness and mortality. All surviving animals were euthanized. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (National Institutes of Health Publication No. 86-23, revised 1985), and used facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Cloning and sequence analysis of the GP gene of EBOV, strain Zaire 95

Total cell monolayers from four 25-cm² flasks of EBOV-infected Vero E6 cells were harvested when CPE became evident. Medium was removed from the flasks and discarded and the infected cell monolayers were lysed by the addition of 1 ml Trizol reagent (Gibco BRL) to each flask. The lysates were removed from the BSL-4 laboratory for all subsequent manipulations. The samples were extracted with chloroform, and RNA was precipitated with isopropanol and recovered by centrifugation. First-strand cDNA synthesis were performed by using the total RNA recovered from one of the flasks and a Superscript cDNA kit (Gibco BRL) according to the manufacturer's directions. Primers for RT-PCR were designed based on the reported sequence of EBOV, strain Zaire 76 (Sanchez et al., 1996), GenBank Accession Nos. U28077 and U23187). Two different forward primers were used which corresponded to nucleotides 5901–5912 or 6037–6054 of the previously reported sequence and which added a BgIII site to the amplified gene to facilitate cloning. The forward primers used were 5'-gatcagatctGCGATGAA-GATTAAAG-3' or 5'-gatcagatctACAATGGGCGTTACAGG-3', with nucleotides added for cloning purposes listed in lowercase letters and EBOV-specific nucleotides listed in uppercase letters. The reverse primer used was 5-gatcagatctCTAAAGACAAATTTG-3'. PCR was performed by using the Expand Long Template PCR System (Boehringer Mannheim). PCR conditions were 1 cycle of 94°C for 3 min, 42°C for 2 min, and 72°C for 5 min; 15 cycles of 94°C for 1 min, 42°C for 2 min, and 72°C for 5 min; 10 cycles of 94°C for 1 min, 42°C for 2 min, and 72°C for 7 min; 1 cycle of 94°C for 1 min, 42°C for 2 min, and 72°C for 10 min. Samples were then held at 4°C until used for cloning into the pCRII plasmid (Invitrogen). After determining orientation of the inserts by restriction digestion, the EBOV GP genes were excised from pCRII by digestion with BgIII and were cloned into the BamHI site of the pRGR7077 (Schmaljohn et al., 1997). The nucleotide sequences of the cloned genes were determined by using dideoxy sequencing of 32P-radiolabeled products according to the directions included with the Sequenase DNA sequencing kit (Amersham).

Cloning and sequence analysis of the NP gene of EBOV

RT-PCR was performed as described above for the GP gene, except that first-strand cDNA was generated by using random hexamers, rather than specific primers. The NP forward and reverse PCR primers, respectively, were 5'-GATGGATTTCCGCTCTC-3' and 5'-CATCAGTGGTGC-3', which correspond to nucleotides 486±485 and 2674±2692 of the previously reported sequence of the 1976 isolate of EBOV (Sanchez et al., 1993). The PCR products were cloned into pCR II plasmid as de-
scribed above for GP and were then excised by restriction digestion with NotI and BamHI and were ligated to the same sites in pWRG7077.

Transient expression assays and gel electrophoresis

Expression of EBOV proteins was first measured by using a transient vaccinia virus/T7 polymerase assay (Fuerst et al., 1986) and the pCRII construct. The assay was performed with modifications reported previously (Iacono-Connors et al., 1996). Briefly, plasmids were transfected into recombinant vaccinia virus-infected baby hamster kidney cells (m.o.i. 10) in a six-well cell culture plate by using Lipofectin reagent (Gibco-BRL). The cells were radiolabeled from 20 to 24 h postinfection with 100 μCi/ml of [35S]Promix (methionine and cysteine) (Amersham) and lysed with a buffer consisting of 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl, 4% Zwittergent 3-14 (Calbiochem-Behring), and protease inhibitors (Boehringer Mannheim). Cell nuclei were removed by centrifugation for 5 min at 12,000 g in a microcentrifuge. An aliquot (100 μl) of each supernatant was mixed with 5 μl of a polyclonal hyperimmune guinea pig serum to EBOV. After incubation on ice overnight, 100 μl of 50% Protein A–Sepharose (Sigma) in lysis buffer was added to each tube, and the samples were shaken at 4°C for 30 min. The Sepharose beads were recovered by centrifugation in a microcentrifuge and were washed three times with lysis buffer and one time with 10 mM Tris·HCl, pH 8.0. The beads were then boiled for 2 min in protein sample buffer and analyzed by SDS·polyacrylamide gel electrophoresis (PAGE) as described previously (Arikawa et al., 1989).

Transient expression assays of the pWRG7077 constructs were performed by transfection of COS cells with 5 μg of each plasmid containing one of the two EBOV GP genes or with 5 μg of pWRG7077 with no insert as described previously (Schmaljohn et al., 1997). The cells were radiolabeled from 24 to 28 h after infection, lysed, and analyzed by gel electrophoresis as described above.

Preparation of gene gun cartridges, immunization, and challenge of mice

Plasmid DNA was precipitated onto gold beads as described previously (Eisenbraun et al., 1993). The gold beads were approximately 2 μm in diameter and were mixed with approximately 0.5±0.1 μg of DNA/mg gold. The DNA-coated gold particles were dried on the inside walls of Tefzel tubing, which was then cut into 0.5-in. sections. Each cartridge contained approximately 0.5 mg of gold (dry weight) with net loads of 0.25±0.5 μg of DNA (measured by fluorometric assays of eluted DNA or estimated by gel electrophoresis and comparison to ethidium bromide-stained standards). The Powderject-XR gene delivery device (D. McCabe, inventor. Agracetus, Inc., assignee; PCT patent WO 95/19799, July, 27, 1995) was used to achieve intracellular inoculation of epidermal cells of BALB/c mice (approximately 6 to 8 weeks old) with approximately 0.5±1 μg of DNA (Pertmer et al., 1995). Blood samples were collected from the retroorbital sinuses of the mice at various times after vaccination. For challenge studies, the mice were transferred to a BSL-4 containment area and inoculated IP with approximately 30 times the amount of mouse-adapted EBOV previously determined to cause death in 50% of adult BALB/c mice (30 LD50). This dose equaled 1 plaque-forming unit on Vero E6 cells and 30 virions as measured by electron microscopy. Mice were observed daily for signs of illness and for death. In one experiment, groups of 10 ear-tagged vaccinated or naive mice were challenged with virus, and the first 4 mice from each group were terminally exsanguinated on day 3 postinfection. All surviving mice were terminally exsanguinated on day 5 (6 vaccinated and 5 naive control mice).

Plaque assay and plaque reduction neutralization test (PRNT50)

To quantitate viral infectivity, virus stocks or whole blood samples from mice were serially diluted in growth medium and then were allowed to adsorb to confluent Vero E6 cells in 12-well cell culture plates for 1 h at 37°C. The cells were overlaid with agarose diluted in cell culture medium and incubated at 37°C for 6 more days (Bray et al., 1998). A 1:5000 dilution of neutral red in buffered saline solution was then added to the wells and plaques were counted the following day.

To measure neutralizing antibodies, aliquots of EBOV in growth medium were mixed with serial dilutions of pre- or postchallenge mouse sera and incubated at 34°C for 1 h. The mixture was used to infect Vero E6 cells, an agarose overlay was applied, and plaques were counted 1 week later. Controls consisted of virus mixed with serial dilutions of a high-titer anti-EBOV serum, or with normal serum, or with medium only. The neutralizing antibody titer was calculated as the reciprocal of the highest dilution of serum which caused a 50% reduction in the number of plaques compared to the normal serum control.

ELISA

To prepare antigen for ELISA, EBOV was purified and inactivated as described previously for MBGV (Hevey et al., 1997). Briefly, supernatants from EBOV-infected cultures of Vero E6 cells were concentrated by polyethylene glycol precipitation. The PEG precipitates were resuspended in buffer and separated by centrifugation in continuous 20±60% sucrose gradients for 4 h at 38,000 rpm in a SW41 rotor (Sorvall). The purified virus was inactivated by irradiation (6 MR, 60Co source) and tested for the absence of infectivity in cell culture before use. ELISA was performed essentially as described previ-
ously (Hevey et al., 1997). Briefly, 96-well polystyrene microwell plates (Dynatech, Vienna, VA) or high-binding RIA/EIA flat-bottom plates (Costar) were coated with 50 μl/well of antigen at a predetermined optimal dilution (1:500 or 1:1000) in PBS. Plates were incubated at 4°C overnight and then nonspecific binding was blocked by the addition of 200 μl/well of 5% powdered nonfat milk in PBS containing 0.02% Tween 20. Test antibodies were diluted in either half-log or fivefold increments. Secondary antibody was horseradish peroxidase (HRPO)-labeled goat anti-mouse IgG antibody (Kirkegaard and Perry Laboratories) and the detector substrates were either 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS; Kirkegaard and Perry) or 3,3′,5,5′-tetramethylbenzidine (TMB, Kirkegaard and Perry Laboratories). Values were read at 405 nm (ABTS) or 450 nm (TMB). Endpoint titers were defined as OD values >0.2 above the mean OD value obtained with the same dilution of serum from control mice.

Transformation and selection of P815 cells

To prepare target cells for chromium release assays, EBOV NP or GP cDNA was cloned into the plasmid vector pCDNA 3.1 (Invitrogen), and the plasmids were used to transform P815 mouse cells. For the GP cell line, approximately 0.5 μg of DNA was introduced into the cells by gene gun inoculation of approximately 300,000 cells suspended in 20 μl of RPMI 1640 medium. For the NP cell line, 5 μg of DNA and 25 μl of Lipofectamine (Gibco-BRL) were used to transfet to was used to transfet cells approximately 10⁶ cells in 0.8 ml of OptiMEM (Gibco-BRL) according to the directions accompanying the Lipofectamine reagent. After incubation in transfection medium for 8 to 24 h, the medium was replaced with RPMI plus G418 in 96-well microtiter plates. Wells were blocked by the addition of ACK lysis buffer (Quality Biologicals, Gaithersburg, MD) and the splenocytes recovered by centrifugation. For in vitro stimulation of the splenocytes, 4 × 10⁵ P815 cells (controls) or P815 cells transformed to express EBOV NP or GP were treated with 01 mg/ml of mitomycin C and were then incubated with 6 × 10⁸ splenocytes per well in 24-well plates in medium containing 10 units/ml rat interleukin-2 (Collaborative Research) for 5 to 7 days. Following stimulation, the splenocytes (effector cells) were collected and plated into 96-well plates at various ratios relative to 40,000 target cells radiolabeled with ⁵¹Cr for 5 to 7 h. Supernatants were collected and counted by using an LKB gamma counter or a Beckman Top Count Instrument. Maximum release was obtained by lysis of control wells with 2% Triton × 100 in PBS. Spontaneous release was determined from target wells to which only medium was added. Lysis was calculated according to the formula: % Lysis = 100% × [average CPM from duplicate test, naive, or control wells] — average CPM for spontaneous release wells]/Average CPM for maximum release wells. Specific lysis was defined as the percentage lysis calculated as above minus the percentage lysis obtained with lymphocytes from mice immunized with an irrelevant plasmid DNA.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical help of Cynthia Kelley and Diane Negley.

REFERENCES


Elliott, L. H., Sanchez, A., Holloway, B. P., Kiley, M. P., and McCormick,


