The filoviruses Ebola virus and Marburg virus (22) cause severe hemorrhagic fever and high mortality in humans. In fatal infections, the host dies with a high viremia within a few days of the onset of symptoms and there is little evidence of any immune response. There are no vaccines or effective treatments for filovirus infection.

We are interested in determining the activity of antibodies (Abs) against filoviruses so that this might be exploited in vaccine design and possibly in prophylactic or therapeutic agents. When immune system-based countermeasures to filoviruses are considered, survivors of infection might provide important information. About 10 to 20% of those infected with Ebola virus antigens was isolated from phage display libraries constructed from RNA from donors who recovered from infection in the 1995 Ebola virus outbreak in Kikwit, Democratic Republic of Congo. Antibodies reactive with nucleoprotein (NP), envelope glycoprotein (GP), and secreted envelope glycoprotein (sGP) were characterized by immunofluorescence and radioimmunoprecipitation assays. Four antibodies reacting strongly with sGP and weakly with GP and two antibodies reacting with NP were not neutralizing. An antibody specific for GP neutralized Ebola virus to 50% at 0.4 μg/ml as the recombinant Fab fragment and to 50% at 0.3 μg/ml (90% at 2.6 μg/ml) as the corresponding whole immunoglobulin G1 molecule. The studies indicate that neutralizing antibodies are produced in infection by Ebola virus although probably at a relatively low frequency. The neutralizing antibody may be useful in vaccine design and as a prophylactic agent against Ebola virus infection.

The extent to which the rodent models are representative of human filovirus infection is not known. Considerable viral adaptation may be involved in the model. For instance, Ebola virus must undergo eightfold serial passage through mice to produce a virus lethal for these animals (4). It is therefore important to carry out studies in nonhuman primates. One detailed study has been carried out to evaluate the efficacy of passively administered antibody in protection against Ebola virus in macaques (13). The Ab used was an immunoglobulin G (IgG) preparation from a horse that had been hyperimmunized with Ebola virus (15, 16) and had a high neutralizing-Ab titer as assessed in a plaque reduction assay. The antibody did delay the onset of clinical symptoms and viremia, but 11 of 12 infected monkeys eventually died. As noted by the authors of that study, the polyclonal equine IgG has a number of limitations, suggesting that it may be valuable to investigate the protective and therapeutic benefit of human monoclonal IgGs. The limitations include the inherently rather low specific activity achievable by passive administration of a polyclonal Ab compared to a monoclonal Ab and the unfavorable pharmacokinetics and diminished effector function activity of an equine IgG in macaques. Human IgGs are very similar to

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macaques IgGs and are expected to show good pharmacokinetics and effector function activity in the macaques (3).

However, although the use of potent neutralizing human Abs to filoviruses could potentially answer a number of questions, it is not clear that such Abs are produced in natural infection as opposed to the hyperimmunization method used to generate equine IgG as described above. Neutralizing-Ab titers in serum of patients recovering from Ebola virus infection are typically low. These could reflect low concentrations of potent neutralizing Abs in serum or higher concentrations of weak neutralizing Abs. The latter are unlikely to be abundant in recovered patients, given the results of the studies with macaques. On the other hand, potent neutralizing Abs would signal potential approaches for vaccine development and might prove useful in prophylactic or therapeutic reagents.

To investigate the Abs produced in Ebola virus infection in humans, we have constructed Ab phage display libraries from donors who recovered from infection in the 1995 Ebola Zaire virus outbreak in Kikwit, Democratic Republic of Congo. Specific Abs have been affinity selected from these libraries on Ebola virus antigens including whole inactivated virions. One anti-GP Fab has been engineered to a whole IgG1 molecule and shown to potently neutralize Ebola virus in vitro. A preliminary account of the feasibility of isolating specific human Abs from Ebola virus infection-convalescent donors appeared previously (18).

MATERIALS AND METHODS

Sample collection and RNA preparation. Bone marrow was obtained from two donors (designated K and L) who recovered from infection with Ebola virus during the 1995 outbreak in Kikwit, Democratic Republic of Congo. Donor K became ill on 4 May 1995 and was hospitalized on 7 May. Donor L became ill on 14 April 1995 and was hospitalized on 19 April. Bone marrow from both donors was drawn on 22 August. Serum samples from each donor were drawn concomitantly. Peripheral blood from 10 donors including donors K and L was drawn as well; these samples were drawn between 5 and 8 July 1995.

Bone marrow of donor K was lysed by vigorous mixing with denaturant solution (4.2 M guanidine hydroxycyanate [Fluka Biochemika, Buchs, Switzerland]; 17 mM sodium N-laurolysarcosine [Sigma, St. Louis, Mo.]; 25 mM trisodium citrate [Sigma]; 50 mM 2-mercaptoethanol [Sigma]), which had been sent to Zaire from the US by the Armed Forces Institute of Pathology (AFIP). Because of difficulties of isolating RNA on-site, we requested that the bone marrow be diluted at least fourfold in the denaturant solution immediately after bone marrow puncture. The samples were held at 4°C and shipped to the United States. Peripheral blood mononuclear cells (PBMC) from donors L and K were isolated from blood by Ficoll-Hypaque centrifugation. On arrival, blood was centrifuged at 1,000 × g for 20 min at 4°C. The RNA pellet was resuspended in 3 ml of denaturant solution and precipitated with 3 ml of cold absolute ethanol at −20°C. After an incubation of 1 h at −20°C with 200 μl of 3 M NaOAc, the precipitate was washed with 1 ml of 70% ethanol, air-dried, and dissolved in RNase-free water.

RNA was prepared by adding 1 ml of 2 M sodium acetate (pH 4.0) to each 10-ml portion of lysate. The samples were extracted with 10 ml of acidic phenol and 10 ml of chloroform-10 ml of I-saturated with 0.1 M citrate buffer (pH 4.3) [Sigma]) and 2 ml of a chloroform-10-ml portion of lysate. The samples were extracted with 10 ml of acidic phenol and 10 ml of chloroform and precipitated with 20 ml of I-saturated ethanol and 2 ml of 3 M NaOAc and centrifuged for 1 h at 37°C. The carbenicillin concentration was increased to 50 μg/ml and neutralized with 2 M Tris–1% normal goat serum (NGS)–IgG-coupled Sepharose 4B (Pharmacia) after the last wash step. The samples were then digested with Nhel and SpeI restriction endonucleases and religated to excise the cpll gene and obtain plasmids producing soluble Abs.

Screening of soluble Fab fragments. Microtiter wells were coated overnight at 4°C with the two Ebola antigens used for panning and a control antigen, ovalbumin (4 μg/ml) (Pierce, Rockford, Ill.). Soluble Fabbs were tested by an enzyme-linked immunosorbent assay (ELISA) as described previously (18). DNA sequencing. Fabbs were analyzed for their DNA sequence with a 373A or 377A automated DNA sequencer (ABI, Foster City, Calif.), using a Tag fluorescent dye terminator cycle-sequencing kit (ABI), as described previously (2).

Neutralization assay. To observe the binding of Fabbs to live cells infected with Ebola virus, 10 6 inactivated Vero E6 cells infected with Ebola Zaire (1011 PFU) were added to wells of a 96-well microtiter plate coated with 1:100 sera. Bioassay Safety Level 4 conditions on 16-well chamber slides for 3 to 4 days. Each well was incubated with 10 μl per ml in 1% bovine serum albumin–0.05% NaN3–PBS. To avoid nonspecific Ab uptake by the cells, the wells were incubated on ice for 30 min. The wells were then washed with PBS and air dried, and the cells were γ-irradiated and fixed in acetone for 5 min. The cells were then incubated for 1 h at 37°C with a 1:200 dilution of fluorescein isothiocyanate-coupled goat anti-human IgG Fab (Jackson). In PBS–1% normal goat serum, the cells were washed with PBS–1% NGS. For the neutralization assay, the cells were washed and blocked with 4% nonfat dry milk (Bio-Rad) for 1 h at 37°C. The wells were air-dried, and the cells were examined by immunofluorescence. Immunofluorescence with fixed cells was performed in a similar manner except that cells were fixed and permeabilized for 5 min in acetone before being incubated with Fabbs (18).

Immunofluorescence. To observe the binding of Fabbs to live cells infected with Ebola virus, 10 6 inactivated Vero E6 cells infected with Ebola Zaire (1011 PFU) were added to wells of a 96-well microtiter plate coated with 1:100 sera. Bioassay Safety Level 4 conditions on 16-well chamber slides for 3 to 4 days. Each well was incubated with 10 μl per ml in 1% bovine serum albumin–0.05% NaN3–PBS. To avoid nonspecific Ab uptake by the cells, the wells were incubated on ice for 30 min. The wells were then washed with PBS and air dried, and the cells were γ-irradiated and fixed in acetone for 5 min. The cells were then incubated for 1 h at 37°C with a 1:200 dilution of fluorescein isothiocyanate-coupled goat anti-human IgG Fab (Jackson). In PBS–1% normal goat serum, the cells were washed with PBS–1% NGS. For the neutralization assay, the cells were washed and blocked with 4% nonfat dry milk (Bio-Rad) for 1 h at 37°C. The wells were air-dried, and the cells were examined by immunofluorescence. Immunofluorescence with fixed cells was performed in a similar manner except that cells were fixed and permeabilized for 5 min in acetone before being incubated with Fabbs (18).
Infectious virus remaining in the virus-Ab mixture was quantitated by counting PFU on Vero E6 cell monolayers. A 0.2-ml volume of each mixture was adsorbed to cells grown in 10-cm² wells of plastic plates (37°C for 1 h). Each mixture was assayed in two wells. Following adsorption, the cells were overlaid with 2 ml of Eagle’s minimal essential medium containing 5% fetal bovine serum, 25 mM HEPES buffer, 50 μg of gentamicin per ml, and 1% agarose. The cells were incubated at 37°C in a humidified CO₂ incubator until plaques were visible under incubation, 2 ml of neutral red (1:6,000 final concentration) was added to each well, and the plaques were counted after an additional 24-h incubation (14).

Preparation of IgG1 KZ52. To convert Fab KZ52 to a whole IgG molecule, the heavy-chain variable gene fragment and the light-chain gene of KZ52 were cloned into a eukaryotic expression vector containing the human IgG1 constant-region gene and the protein was expressed in CHO cells as described previously (6). IgG1 KZ52 was purified by protein A column chromatography (Pharmacia).

RESULTS

Ab library characterization. Two IgG1 κ libraries were constructed from bone marrow of convalescent donors (K and L) and contained a diversity of 6 × 10⁶ and 2.2 × 10⁷ clones, respectively. IgG1 κ and IgG1 λ libraries (designated E10κ and E10λ, respectively) constructed from pooled RNA of peripheral-blood lymphocytes from 10 convalescent donors including donors K and L both contained a diversity of 5 × 10⁶ clones. Isolation of specific Fabs from the libraries by affinity selection against Ebola antigens. The libraries were panned against γ-irradiated preparations of whole virions (Ebola Zaire virus) and crude supernatants from cultures of infected cells. The former contained all the viral structural proteins, and the latter was greatly enriched for secreted GP (sGP) (see Fig. 3). Specific Fabs were identified by a strong ELISA reactivity with the selecting antigen and a low reactivity with a control antigen (ovalbumin) (Fig. 1).

Positive clones were sequenced to reveal relatedness. Table 1 shows that from library K two distinct Fabs (indicated by the prefix KZ) were isolated by selection against the virion preparation and four were isolated by selection against the infected-cell supernatant preparation (prefix KS). However, the clone KZ52 selected by panning against the virion preparation was identical in sequence to the clone KS56 isolated by panning against the supernatant preparation. Two distinct Fabs, with identical heavy-chain but different light-chain sequences, were isolated from library L by panning against the supernatant preparation (LS4) and the virion preparation (LZ51). A single Fab was selected from the pooled PBMC libraries (E10κ and E10λ) by panning against the virion preparation.

The reactivity of specific Fabs against viral preparations other than the selecting antigen was explored. Three distinct reactivity profiles were apparent as exemplified by the Fabs in Fig. 1. Fabs KZ51 and ELZ510, obtained by panning against the virion preparation, showed no reactivity with the supernatant preparation. Fab KZ52, also obtained by panning against the virion preparation, had a unique reactivity pattern. In addition, the reactivity of specific Fabs against viral preparations other than the selecting antigen was explored. Three distinct reactivity profiles were apparent as exemplified by the Fabs in Fig. 1. Fabs KZ51 and ELZ510, obtained by panning against the virion preparation, showed no reactivity with the supernatant preparation. Fab KZ52, also obtained by panning against the virion preparation, had a unique reactivity pattern. In addition, the reactivity of specific Fabs against viral preparations other than the selecting antigen was explored. Three distinct reactivity profiles were apparent as exemplified by the Fabs in Fig. 1. Fabs KZ51 and ELZ510, obtained by panning against the virion preparation, showed no reactivity with the supernatant preparation. Fab KZ52, also obtained by panning against the virion preparation, had a unique reactivity pattern.

<table>
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<tr>
<th>Fab</th>
<th>HCDR3 sequence</th>
<th>LCDR3 sequence</th>
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<tr>
<td>KZ51</td>
<td>EVVVVTPAPTNYFYYYMDV</td>
<td>AHRRGWPLS</td>
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<tr>
<td>KZ52</td>
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<td>NEMSYDITGPGDYLDS</td>
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<td>KYSRRDLV</td>
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<td>RGSITLHREGNWFDP</td>
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<td>QOANTFPP</td>
</tr>
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<td>TSLFAEVLMDVF DI</td>
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<tr>
<td>Library E10; IgG1 λ</td>
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<tr>
<td>ELZ510</td>
<td>GYCSSTSCPLFD</td>
<td>ATWADSLGYY</td>
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* HCDR3 and LCDR3 indicate heavy-chain and light-chain complementarity-determining region, respectively.

<table>
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<tr>
<th>Fab</th>
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<th>Primary antigen specificity</th>
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<td>+ + + +</td>
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* ND, not determined.
dition to virion binding, it showed significant cross-reactivity with the supernatant preparation. Fabs LS4, KS14, KS518, and LZ51, obtained by panning against the supernatant preparation, showed some weak cross-reactivity with the virion preparation. These profiles can be readily interpreted in terms of the antibody specificities determined below and summarized in Table 2.

**Binding of Fabs to live and fixed Ebola virus-infected cells as confirmed by immunofluorescence.** The specificity of the selected Fabs for Ebola antigens was confirmed by immunofluorescence to detect Fab binding to live and fixed Ebola virus-infected cells. All Fabs reacted with fixed infected cells but not with uninfected control cells. Four reactivity patterns with live Ebola virus-infected cells were observed among the Fabs tested. Fab KZ52 reacted strongly with live infected cells (Fig. 2A), giving a staining pattern that was indistinguishable from that of human convalescent-phase serum. Fabs LZ51, LS4, KS518, and KS5 showed a weak “intercellular” staining pattern of live infected cells (Fig. 2B), and LZ51 showed a spotty cytoplasmic pattern on fixed infected cells, suggestive of Golgi-like staining (Fig. 2E). Fabs ELZ510 and KZ51 reacted only with a few live infected cells (less than 1 per field of 100 [Fig. 2C]), which may represent disrupted cells. Consistent with this, these Fabs did react well with fixed cells and showed a distinctive cytoplasmic staining pattern (Fig. 2F). Fab KS14 did not stain live infected cells but did stain fixed infected cells (data not shown).

**RIPA of Fab reactivity.** Two types of antigens were used in RIPA: cell lysates which contained all the structural viral proteins (Fig. 3A, lane 10) and a crude supernatant antigen rich in...
SGP but also containing virions (26). Fabs of three broad specificities were identified (summarized in Table 2): those that reacted with NP, those that reacted primarily with SGP, and one that reacted with GP. The RIPA reactivity of Fabs KZ51 and ELZ510 suggested that they were specific to NP in that they specifically precipitated a band of 97 kDa both from Ebola virus-infected cell lysates and from crude supernatants labeled with [35S]Cys-[35S]Met but not from mock-infected cells (Fig. 3A, lanes 1 to 6). This 97-kDa protein was not observed when [3H]glucosamine-labeled antigens were used (results not shown). KZ51 immunoprecipitated a 97-kDa band from infected-cell lysates of three other Ebola virus subtypes (Reston, Sudan, and Ivory Coast) in addition to the Zaire subtype. ELZ510 showed reactivity only with the Ivory Coast subtype in addition to the Zaire virus (data not shown).

Fabs KZ52, KS518, and LZ51 immunoprecipitated a 120-kDa band corresponding to GP1 from [3H]glucosamine-labeled Ebola virus-infected cell lysates but not from mock-infected controls (Fig. 3B). Similarly, Fab KS14 immunoprecipitated a 120-kDa band corresponding to GP1 from [35S]Cys-[35S]Met-labeled Ebola virus cell lysate. However, whereas KZ52 precipitated a strong band of 120 kDa from infected-cell supernatants (rich in soluble GP and virions), the other three Fabs immunoprecipitated a strong band in the 50-kDa region corresponding to SGP (Fig. 3B; Fig. 3A, lane 8). A longer exposure of the autoradiograms indicated a band at about 24 kDa, corresponding to GP2 from this supernatant (lanes 5 and 8), with only a faint band at 120 kDa. (C) Longer-exposure autoradiograms obtained to reveal the immunoprecipitation of GP2. Exposure of autoradiograms for 2.5 months (compared to 1 week for panel B) reveals immunoprecipitation of a band corresponding to GP2 by Fabs KZ52, KS518, and LZ51 from Ebola virus-infected cell supernatants (lanes 1, 4, and 7, respectively) and from crude infected-cell supernatants (lanes 2, 5, and 8, respectively). Lanes 3, 6, and 9 are uninfected-cell supernatant controls.

Neutralizing activity of Ebola virus-specific Fabs. Fabs were tested for their ability to neutralize the virus in a plaque re-

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**FIG. 3.** RIPA to show the specificities of anti-Ebola Zaire virus recombinant Fabs. (A) Immunoprecipitations are shown for Fab KZ51 (lanes 1 to 3), Fab ELZ510 (lanes 4 to 6), Fab KS14 (lanes 7 and 8), and convalescent-phase human serum (lanes 10 and 11). For reference, a [35S]Cys-[35S]Met-labeled virion preparation is shown in lane 12. The first lane shown for each Fab is the immunoprecipitate from [35S]Cys-[35S]Met-labeled Ebola virus-infected cell lysate, the second lane is from [35S]labeled crude Ebola virus supernatant, and the third lane is from [35S]-labeled lysates from uninfected cells. KZ51 and ELZ510 precipitated NP (97 kDa) from [35S]-labeled Ebola virus-infected cell lysate (lanes 1 and 4, respectively) and [35S]-labeled crude Ebola virus supernatant (lanes 2 and 5, respectively). (B) Immunoprecipitations are shown for Fab KZ52 (lanes 1 to 3), Fab KS518 (lanes 4 to 6), and Fab LZ51 (lanes 7 to 9). For each Fab, immunoprecipitations are shown from [3H]glucosamine-labeled Ebola virus-infected cell lysate, [3H]-labeled Ebola virus supernatants, and [3H]-labeled uninfected cell lysates, respectively. KZ52, KS518, and LZ51 immunoprecipitated a band of 120 kDa corresponding to GP1 from [3H]glucosamine-labeled Ebola virus-infected cell lysate (lanes 1, 4, and 7, respectively). KZ52 also immunoprecipitated a band of 120 kDa from [3H]glucosamine-labeled Ebola virus supernatant (lane 2). The other two Fabs predominantly immunoprecipitated a band of 50 kDa, corresponding to SGP from this supernatant (lanes 5 and 8), with only a faint band at 120 kDa. (C) Longer-exposure autoradiograms obtained to reveal the immunoprecipitation of GP2. Exposure of autoradiograms for 2.5 months (compared to 1 week for panel B) reveals immunoprecipitation of a band corresponding to GP2 by Fabs KZ52, KS518, and LZ51 from Ebola virus-infected cell supernatants (lanes 1, 4, and 7, respectively) and from crude infected-cell supernatants (lanes 2, 5, and 8, respectively). Lanes 3, 6, and 9 are uninfected-cell supernatant controls.
assay as described in the text. Neutralization of Ebola Zaire 1995 virus was measured in a plaque reduction assay as described in the text. Neutralization of Ebola Zaire 1995 virus was measured in a plaque reduction assay as described in the text.

One Ab, KZ52, showed strong reactivity with GP and no reactivity with sGP. This Ab stained live infected cells particularly strongly and neutralized the virus effectively at nanomolar concentrations. It may have been elicited by virion-bound GP or alternately by secreted or shed GP1, as has been recently described in experiments performed under tissue culture conditions (30). The activity of KZ52 establishes the principle that Abs elicited in natural infection can neutralize a filovirus. The poor neutralization of Ebola virus by convalescent-phase sera (20), however, would indicate that such Abs are probably produced at relatively low frequency. By comparison with other viruses, the potency of neutralization of KZ52 is within the range that may lead to protection in passive-immunization studies. A dose of 10 mg/kg would produce a concentration of Ab in serum 40-fold higher than the 90% in vitro neutralization titer; alternately, a 1:40 dilution of serum should produce 90% neutralization. This is the type of efficacy that has been effective for other viruses (9, 17, 23). Passive-immunization studies in rodents and macaques will reveal whether Ebola virus is typical in this regard.

ACKNOWLEDGMENTS

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