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 Zaire virus in the 1976 and 1995 outbreaks survived, but it is not clear to what degree the immune system was involved in their recovery (7, 8, 21). Generally, neutralizing Abs are almost certainly not important because they appear late in the convalescent period and then at very low to insignificant titers (22). Cell-mediated immunity may be crucial, but this is unproven. Transfusion of convalescent-phase whole blood to infected patients in the 1995 Ebola virus outbreak in Kikwit, Democratic Republic of Congo, was anecdotally described as conferring increased survival on treated patients, but other explanations for the survival of these patients have been proposed (18a, 20, 24). There are no reports of immunity to Ebola virus infection after a primary infection.

Rodent models of filovirus infection have been developed and used particularly to investigate immune system correlates of protection. Passive transfer of neutralizing Abs protects guinea pigs from Ebola virus and Marburg virus infection (11, 12). Vaccination with recombinant vaccinia virus expressing Ebola virus glycoprotein (GP) confers partial protection in guinea pigs that is not observed with constructs expressing other Ebola virus proteins (10). These studies imply an important role for antibody in protection against filovirus challenge. Other studies suggest that cell-mediated immunity is important. DNA vaccination with constructs expressing either GP or nucleocapsid protein (NP) protects mice from lethal challenge with Ebola virus (27). Protection of guinea pigs by DNA vaccination was correlated with antibody and cell mediated responses to GP (32).

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 IgG preparation from a horse that had been hyperimmunized with Ebola virus 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
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 weakly neutralizing Abs. The latter are unlikely to be as effective against the virus, given the results of the studies with macaques. On the other hand, potent neutralizing Abs would signal potential applications 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-lauroylsarcosine [Sigma, St. Louis, Mo.], 25 mM trisodium citrate [Sigma], 50 mM 2-mercaptoethanol [Sigma]), which had been sent to Zaire in 10-ml aliquots in 50-ml centrifuge tubes (Corning, Corning, N.Y.). Bone marrow of donor L was immediately frozen in dry ice after aspiration and then shipped to the United States. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and lysed by vigorous mixing with denaturant solution as described for the bone marrow above. Bone marrow from donor L was immediately frozen in dry ice after aspiration and then shipped to the United States on dry ice. This bone marrow was thawed directly in denaturant solution in a laminar-flow biosafety cabinet with the usual precautions.

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 (saturated with 0.1 M citrate buffer [pH 4.3] [Sigma]) and 2 ml of a chloroform-isooamyl alcohol mixture (24:1). After being incubated on ice for 15 min, the samples were centrifuged at 10,000 g for 20 min at 4°C. RNA was precipitated from the supernatant by the addition of 40 g of glycogen (Boehringer Mannheim, Indianapolis, Ind.) and 15 ml of 2-propanol (Sigma), overnight incubation at −20°C, and centrifugation at 10,000 g for 20 min at 4°C. The RNA pellet was redissolved in 3 ml of denaturant solution and precipitated for 3 h at −20°C after the addition of an equal volume of 2-propanol. RNA was pelleted in a microcentrifuge, washed twice with 70% ethanol, and resuspended in diethylpyrocarbonate-treated water.

Library construction. First-strand cDNA was prepared by priming with oligo d(T) with a cDNA kit (Boehringer Mannheim) as recommended by the manufacturer. The IgG1 Fd region and whole x and y light chains were then amplified by PCR as described previously (19). The cDNA fragment of each Fab library was cloned into the phagemid pComb3H (32) as described previously (5). Briefly, the light-chain and heavy-chain PCR fragments were mixed in equimolar amounts and ligated into the SpeI and XhoI restriction sites of the phagemid, respectively. Ligation products were ethanol precipitated and electroporated into Escherichia coli XL1-Blue cells (Strategene, La Jolla, Calif.). The transformed E. coli cultures were grown in SOC medium and then in SB medium containing 10 µg of tetracycline per ml and 20 µg of carbenicillin per ml, each for 1 h at 37°C. The carbenicillin concentration was increased to 50 µg/ml, and after the culture had grown for 1 h, phage particles were harvested by centrifugation at 4,000 × g for 20 min and the supernatant of VCS-M13 helper phage (5 × 1011 PFU). After an additional 2 h of culture, kanamycin was added to a concentration of 50 µg/ml and the culture was grown overnight at 30°C. Phage was recovered from the cultures by removing bacteria by centrifugation at 4,000 × g for 20 min and precipitation of phage from the supernatant by addition of 4% polyethylene glycol and 0.5 M NaCl and incubation of the mixture on ice for 30 min. After centrifugation, phage pellets were resuspended in 500 µl of phosphate-buffered saline (PBS—4% nonfat dry milk [Bio-Rad, Hercules, Calif.]) and centrifuged at 5 × 106 rads for 1 h at 37°C. The milk solution was shaken out, phage solution was added to each well, and the mixture was incubated for 2 h at 37°C on a rocker platform. The phage solution was removed, and the wells were washed. Bound phage was eluted with glycine buffer (pH 2.2) and neutralized with 2 M Tris base. Eluted phage was reassayed for the next round of panning as previously described (5). The libraries were panned for four or five consecutive rounds with increasing washing stringency (2, 5, and 10 wash steps thereafter, each consisting of a 5-min incubation and vigorous mixing of the lid). PhageDb (33) was solubilized after the last wash step and digested with NheI and SpeI restriction endonucleases and religated to excise the cpIII gene and obtain plasmids producing soluble Fabs.

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 Fabs were tested by an enzyme-linked immunosorbent assay (ELISA) as described previously (18).

DNA sequencing. Fabs were analyzed for their DNA sequence with a 373A or 377A automated DNA sequencer (ABI, Foster City, Calif.), using a Tag fluorescent dideoxy terminator cycle-sequencing kit (ABI), as described previously (2).

Neutralization assay. All dilutions were made in Eagle’s minimal essential medium (EMEM) and 2% FBS. Neutralizing Abs were tested against 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).

VOL. 73, 1999 NEUTRALIZING HUMAN MONOCLONAL Ab AGAINST EBOLA VIRUS 6025
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 μM of gentamicin per ml, and 1% agarose. The cells were incubated at 37°C in a humidified CO2 incubator until plaques were visible under ultraviolet light.

**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 × 10⁶ clones, respectively. IgG1 κ and IgG1 λ libraries (designated E10κ and E10λ, respectively) were constructed from pooled RNA of peripheral-blood lymphocytes from 10 convalescent donors including donors K and L both contained a diversity of 5 × 10⁶ and contained a diversity of 6 × 10⁶ clones.

**Isolation of specific Fabs from the libraries by affinity selection against Ebola antigens.** The libraries were panned against the supernatant preparation. Two distinct Fabs, identified by their unique reactivity pattern, were isolated from library E10 by panning 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, 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.

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### Table 1. CDR3 sequences of Fabs to Ebola Zaire virus

<table>
<thead>
<tr>
<th>Fab</th>
<th>HCDR3 sequence</th>
<th>LCDR3 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Library K; IgG1 κ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KZ51</td>
<td>EGVPRAGYSMDAVFDI</td>
<td>AHRGGWPLS</td>
</tr>
<tr>
<td>KS56, KZ52</td>
<td>NEMSYYLGSPGVDYLD</td>
<td>OQQYYAPP</td>
</tr>
<tr>
<td>KS14</td>
<td>KYSRSLDVL</td>
<td>OQQYFAVTVT</td>
</tr>
<tr>
<td>KS5</td>
<td>RGSITLHREGNWFDP</td>
<td>QQGDNWPPT</td>
</tr>
<tr>
<td>KS1518</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Library L; IgG1 κ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS4</td>
<td>TLSFAEVLMDVFDI</td>
<td>MPGTHWPPT</td>
</tr>
<tr>
<td>LZ51</td>
<td>TLSFAEVLMDVFDI</td>
<td>QOANTFPPT</td>
</tr>
<tr>
<td>Library E10; IgG1 λ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELZ510</td>
<td>GYCSSTSCPLFDS</td>
<td>ATWADLSGLV</td>
</tr>
</tbody>
</table>

* HCDR3 and LCDR3 indicate heavy-chain and light-chain complementarity-determining region, respectively.

### Table 2. Immunofluorescence and immunoprecipitation of Ebola virus-specific Fabs

<table>
<thead>
<tr>
<th>Fab</th>
<th>Immuno-fluorescence</th>
<th>Specificity deduced from immunoprecipitation with:</th>
<th>Primary antigen specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live</td>
<td>Fixed</td>
<td>Viral lysates</td>
</tr>
<tr>
<td>KZ51</td>
<td>±</td>
<td>±</td>
<td>NP</td>
</tr>
<tr>
<td>ELZ510</td>
<td>+</td>
<td>+</td>
<td>NP</td>
</tr>
<tr>
<td>KZ52</td>
<td>+++</td>
<td>++</td>
<td>GP</td>
</tr>
<tr>
<td>KS14</td>
<td>−</td>
<td>++</td>
<td>sGP &gt;&gt; GP</td>
</tr>
<tr>
<td>KS5</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>KS1518</td>
<td>−</td>
<td>−</td>
<td>GP</td>
</tr>
<tr>
<td>LS4</td>
<td>++</td>
<td>+</td>
<td>sGP &gt;&gt; GP</td>
</tr>
<tr>
<td>LZ51</td>
<td>++</td>
<td>+</td>
<td>sGP &gt;&gt; GP</td>
</tr>
</tbody>
</table>

* 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

FIG. 2. Reactivity of selected anti-Ebola virus Fabs with live and fixed Ebola virus-infected Vero E6 cells shown by immunofluorescence. Staining is shown for Fab KZ52 on live (A) and fixed (D) Ebola virus-infected cells, for Fab LZ51 on live (B) and fixed (E) Ebola virus-infected cells, and for ELZ510 on live (C) and fixed (F) Ebola virus-infected cells. No binding to uninfected Vero E6 cells was observed (data not shown).
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 in mock-infected cells (Fig. 3A, lanes 1 to 6). 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 [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.

Neutralizing activity of Ebola virus-specific Fabs. Fabs were tested for their ability to neutralize the virus in a plaque re-
Neutralization of Ebola Zaire 1995 virus was measured in a plaque reduction assay as described in the text. KZ52 showed 50% neutralization at 0.3 μg/ml (17 nM). A Fab KZ52 was then engineered to a whole human IgG1 molecule and expressed in CHO cells, and the neutralization assay was repeated. As shown in Fig. 4, whole IgG1 KZ52 neutralized about fourfold more effectively than Fab KZ52: 50% at 0.3 μg/ml (2 nM) and 90% at 2.6 μg/ml (17 nM). A control antibody, IgG1 b12 (anti-HIV gp120), showed no inhibition in the number of plaques (data not shown).

**DISCUSSION**

A panel of human monoclonal Abs to Ebola Zaire virus has been generated and characterized in this study, and these Abs reveal aspects of the humoral response to the virus. Two Abs (KZ51 and ELZ510) that immunoprecipitate NP from viral preparations were isolated. These Abs react with fixed but not live infected cells. One of the Abs cross-reacts with NP from four different Ebola virus subtypes, and its binding to NP was inhibited by 10 of 10 Ebola Zaire virus-positive sera tested (data not shown). This suggests that the Ab recognizes a conserved immunodominant epitope on NP and that it may be useful in a competition format in determining seropositivity. For example, the host species of Ebola virus is not known, and a major problem is the availability of detection reagents for a diverse species set. An assay in which test sera compete with an Ab to a conserved immunodominant viral epitope could circumvent this problem.

The remaining Abs were reactive with GP and/or sGP. These two proteins result from unconventional features of GP gene organization and transcription. The primary gene product is sGP, which is encoded in a single reading frame (0 frame). GP is encoded in two reading frames (0 and −1 frames), and expression of GP occurs only when the two frames are connected through a transcriptional editing event (25, 28). Recent studies (26, 29, 30) have revealed that GP and sGP are structurally distinct. Maturation of GP involves cleavage by the enzyme furin into two glycoproteins (GP1 and GP2) which are linked by disulfide bonding. Mature GP is composed of trimers of GP1-GP2 heterodimers. On the other hand, sGP is secreted from infected cells almost exclusively in the form of a homodimer linked by a disulfide bond. Most of the Abs generated here showed a strong reactivity with sGP and a weak reactivity with GP. All of these Abs reacted with fixed infected cells but to various degrees with live infected cells. It seems likely that these Abs were elicited by sGP, which, because of its abundance, could be expected to be a major immunogen during natural infection. The cross-reactivity with GP, albeit relatively weak, suggests that there are related structural elements between the two proteins. This may be detrimental to the development of an optimal antibody response to mature GP, since abundant B cells expressing Ig receptor for sGP could compete for binding to mature GP. Activation of these B cells would produce an Ab of only moderate affinity for mature GP and therefore probably with only weak binding to virions.

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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