Supplemental Material can be found at:
http://www.jbc.org/content/suppl/2012/05/29/M112.364075.DC1.html

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 30, pp. 25203–25215, July 20, 2012
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Synthetic Human Monoclonal Antibodies toward Staphylococcal Enterotoxin B (SEB) Protective against Toxic Shock Syndrome*[S]

Received for publication, March 21, 2012, and in revised form, May 4, 2012 Published, JBC Papers in Press, May 29, 2012, DOI 10.1074/jbc.M112.364075

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Background: SEB is a potent superantigen that can cause toxic shock in humans and can be incapacitating and lethal when used as a bioweapon.
Results: Therapeutic administration of highly affinity-matured synthetic anti-SEB human monoclonal IgGs protects mice from lethal SEB challenge.
Conclusion: Affinity maturation can enhance neutralizing efficacy and lead to successful postexposure therapeutics.
Significance: No vaccines or therapeutics are currently available against SEB.

Staphylococcal enterotoxin B (SEB) is a potent toxin that can cause toxic shock syndrome and act as a lethal and incapacitating agent when used as a bioweapon. There are currently no vaccines or immunotherapeutics available against this toxin. Using phage display technology, human antigen-binding fragments (Fabs) were selected against SEB, and proteins were produced in *Escherichia coli* cells and characterized for their binding affinity and their toxin neutralizing activity *in vitro* and *in vivo*. Highly protective Fabs were converted into full-length IgGs and produced in mammalian cells. Additionally, the production of anti-SEB antibodies was explored in the *Nicotiana benthamiana* plant expression system. Affinity maturation was performed to produce optimized lead anti-SEB antibody candidates with subnanomolar affinities. IgGs produced in *N. benthamiana* showed characteristics comparable with those of counterparts produced in mammalian cells. IgGs were tested for their therapeutic efficacy in the mouse toxic shock model using different challenge doses of SEB and a treatment with 200 μg of IgGs 1 h after SEB challenge. The lead candidates displayed full protection from lethal challenge over a wide range of SEB challenge doses. Furthermore, mice that were treated with anti-SEB IgG had significantly lower IFNγ and IL-2 levels in serum compared with mock-treated mice. In summary, these anti-SEB monoclonal antibodies represent excellent therapeutic candidates for further preclinical and clinical development.

Staphylococcus aureus is a formidable Gram-positive human pathogen that causes a wide range of infections from skin and soft tissue infections to life-threatening diseases like endocarditis, sepsis, pneumonia, and toxic shock (1). The pathogenicity of *S. aureus* is dependent on numerous virulence factors, including cell surface proteins and polysaccharides as well as secreted toxins. The latter cause tissue damage, promote bacterial dissemination and metastatic growth in distant organs, and enable the pathogen to evade the host innate immune response (2, 3).

A major group of these toxins includes staphylococcal superantigens (SAgs),[4] consisting of toxic shock syndrome toxin 1 (TSST-1) and staphylococcal enterotoxins. Staphylococcal enterotoxins and TSST-1 bind to human class II major histocompatibility complex (MHC) on antigen-presenting cells and certain subsets of T cell receptor on T lymphocytes (4). This peptide-independent cross-linking results in massive stimulation of up to 30% of lymphocytes triggering a cytokine storm that can lead to toxic shock syndrome (TSS) (5, 6). TSS can be incapacitating at lower doses of SAgs or lead to multiorgan failure and death at higher doses (6–8). Staphylococcal enterotoxins also cause gastroenteritis and food poisoning by a mechanism that is not fully understood. Most virulent strains of *S. aureus* produce one or more SAgs, and these toxins are believed to play a major role in immune evasion by this pathogen during the course of infection (6). SAgs are also produced by Group A streptococcus, and these SAgs cause the more common streptococcal TSS (9).

* This work was supported, in whole or in part, by National Institutes of Health, NIAID, Grant U01 AI078023-05 (to Integrated BioTherapeutics, Inc.).
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[4] The abbreviations used are: SAg, staphylococcal superantigen; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin 1; SEA, SEB, SEC, SED, and SEK, staphylococcal enterotoxin A, B, C, D, and K, respectively; CDR, complementarity-determining region; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; mIgG, mouse IgG; TNA, toxin neutralization assay; PBMC, peripheral blood mononuclear cell.

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Staphylococcal enterotoxin B (SEB) is one of the most potent enterotoxins involved in a large number of non-menstrual TSS cases as well as a major mediator of staphylococcal food poisoning (6, 7). However, the major source of interest in SEB stems from the potential for this toxin to be used as an agent of biowarfare or bioterrorism. SEB (then code-named PG) was a major and strategic component of the United States offensive program before the ban on biological weapons in 1972 (8). SEB was especially attractive as a bioweapon because of the ease of production, the fact that much lower doses could be effective compared with chemical agents, and its profound potentiating effect as a component of dual agent bioweapons (10). There is currently renewed concern that this toxin can be used in bioterrorism activities. There is currently no therapeutic available for SEB, and a recombinant SEB vaccine (STEBVax) is in early clinical development.

Intravenous immunoglobulin has been used in treatment of streptococcal TSS with limited success (11, 12). However, there is no evidence that intravenous immunoglobulin can be effective against staphylococcal TSS in the clinic (12). Hyperimmune intravenous immunoglobulin could be produced upon donor stimulation with a recombinant attenuated SEB vaccine because this approach has been successful for several other infectious agents. However, this approach is complicated by the need for maintaining a donor cohort, the high dose needed for protection, manufacturing and safety issues, and cost. Monoclonal antibodies represent an attractive alternative to these traditional treatments because these agents can be produced on a large scale using a reproducible process. Recent advances in phage display technologies have led to generation of highly divergent synthetic antibody libraries that can be used for discovery of human antibodies without the need for lengthy hybridoma antibody production and subsequent humanization of mouse monoclonals (13, 14). In the current study, we report the discovery and characterization of highly effective synthetic human antibody therapeutics for prophylactic and postexposure treatment of SEB-induced disease and lethality.

EXPERIMENTAL PROCEDURES

Bacterial Superantigens and Endotoxin—SAgs SEA, SEB, SEC1 to -3, SED, SEK, TSST-1, SpeA, and SpeC were purchased from Toxin Technology (Sarasota, FL) and reconstituted with deionized water. Toxins were aliquoted and stored at −80 °C until use. According to the manufacturer's certificate of analysis, purity of toxins was >95% as determined by SDS-PAGE. Lipopolysaccharide (LPS; *Escherichia coli* 055:B5) was purchased from List Biological Laboratories, Inc. (Campbell, CA) and reconstituted with PBS prior to use.

Construction of Phage-displayed Fab Libraries—The construction of library F, a synthetic Fab-phage library, has been described.[5] For the first round of affinity maturation, two sublibraries were constructed. The template was constructed by introducing TAA stop codons into CDR-L3 in the phagemid pHP153. Phagemid pHP153 displays an anti-MBP Fab fused to the N terminus of the C-terminal domain of the gene-3 minor coat protein (cP3) and was also used as a template in constructing library F. In addition, amber codon TAG was introduced at the fusion point between the heavy chain and cP3 such that the Fab can be displayed on phage in an amber suppressor host, or it can be expressed as free protein in a non-suppressor strain. The resulting phagemid (pGCl054) was used as the template for a mutagenesis reaction with mutagenic oligonucleotides designed to replace TAA stop codons with degenerate codons at the desired CDR sites, as described (15). In the first sublibrary, mutations were introduced into CDR-L3 only with oligonucleotide L3-a (all oligonucleotides used in this study are described in the supplemental material and listed in supplemental Table S1). In the second sublibrary, mutations were introduced into CDR-L3 and one of the other four CDRs: CDR-L1, CDR-H1, CDR-H2, or CDR-H3. CDR-L3 was mutagenized with oligonucleotide L3-b, which is more conservative compared with L3-a used in the first sublibrary. For the second round of affinity maturation, shotgun scanning combinatorial mutagenesis was performed to scan all residues in CDRs of GCI073 to obtain higher affinity SEB-binding Fabs by fine tuning the interaction interface. Accordingly, three sublibraries were constructed. In the first and second sublibraries, alanine-scanning or homolog-scanning, respectively, was utilized to mutagenize CDRs of clone GCI073. In the third sublibrary, the oligonucleotides used for alanine scanning and homolog scanning were premixed and utilized in site-directed mutagenesis for library construction. These sublibraries were eventually pooled before they were subjected to panning.

Selection and Characterization of Anti-SEB Fabs—Biopanning, direct phage ELISAs, and single-point competitive phage ELISAs were performed as described (16). A brief summary of the procedures follows. Phage particles from the libraries were cycled through rounds of binding selection with SEB coated on 96-well Maxisorp Immunoplates (Fisher) as the capture target. After five rounds of selection, phage particles were produced from individual clones grown in a 96-well format, and the culture supernatants were used in phage ELISAs to detect specific binding clones. A single-point competitive phage ELISA was used to rapidly estimate the affinities of phage-displayed anti-SEB Fabs. Clones that bound to SEB and showed ~50% inhibition in the presence of 10 nM solution phase SEB in single-point competitive phage ELISA were subjected to DNA sequence analysis in initial biopanning from library F. The concentrations of solution phase SEB in single-point competitive phage ELISA were decreased to 5 or 2 nM in the successive two rounds of affinity maturation, respectively. In total, 20 unique clones were obtained from initial panning from library F. Twenty-two and three unique clones were obtained from the first and second round of affinity maturation, respectively. The CDR sequences of these clones are listed in Table 1.

Expression and Purification of Fab Proteins—The Fab expression vector was derived from the phage display phagemid by inserting an amber stop codon upstream of the sequence encoding for cP3. Fab protein was produced by growing the transformed 55244 *E. coli* cells in low phosphate CRAP medium (complete CRAP phosphate-limiting medium: 3.57 g of $(NH_4)2SO_4$, 1.07 g of KCl, 0.71 g of sodium citrate dihydrate, 5.36 g of yeast extract, 5.36 g of hycase SF-Sheffield, pH adjusted

[5] H. Persson, W. Ye, A. Wernimont, J. Adams, R. Lam, and S. S. Sidhu, manuscript in preparation.

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with KOH to 7.3, volume adjusted to 872 ml with deionized H_2O and autoclaved; medium supplemented with 110 ml of 1 M MOPS, pH 7.3, and 11 ml of 50% glucose, 7 ml of 1 M $MgSO_4$ before use) at 30 °C for 24–27 h, as described previously (16). The pellet was then resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0, 0.5 mg/ml lysozyme (Bioshop, Burlington, Canada), 10 units/ml benzonase (EMD Chemicals, Gibbstown, NJ), 10 mM $MgCl_2$) and incubated on ice for 1 h. The crude lysate was spun down, and the supernatant was applied to an rProtein A affinity column (GE Healthcare); the column was washed with 250 column volumes of nonpyrogenic PBS buffer supplemented with 0.1% Triton 114 followed by 100 column volumes of nonpyrogenic PBS buffer for Triton 114 removal. Fab protein was eluted with nonpyrogenic elution buffer (50 mM NaH_2PO_4, 100 mM H_3PO_4, 140 mM NaCl, pH 2.0) and neutralized with nonpyrogenic neutralization buffer (1 M Na_2HPO_4, 140 mM NaCl, pH 8.6). Protein concentrations were determined by Bradford assay (Bio-Rad) using bovine γ-globulin as a standard.

Conversion of Fabs to Full-length Antibodies—The VH and VL sequences of individual anti-SEB Fab clones were PCR-amplified using primer sets described in the supplemental material. The resulting VL and VH cassettes were subcloned into BssHII/BsiWI and BssHII/NheI restriction sites in expression vector pMAZ-IgL or pMAZ-IgH (17), respectively. Mammalian vector pMAZ-IgL contains an expression cassette of the constant region of human κ light chain, and pMAZ-IgH contains an expression cassette of the constant region of human γ1 heavy chain. In both cassettes, transcription is driven from a human cytomegalovirus promoter. Vectors were constructed by following standard molecular cloning protocol, and correct variable region sequences were verified by sequencing.

IgG Production in Mammalian Cells—After initial optimization of conditions at a 125-ml scale, a suspension-adapted HEK293T cell line was cultivated in F17 medium (Invitrogen) in 5-liter shake flasks (Corning Inc.). Cells were transfected at a density of ~1–1.5 × 10^6 cells/ml. For transfection, 5 μg of DNA was combined with 25-kDa linear polyethyleneimine (Polysciences, Inc.) and then added to the cells. Cells were fed ~24 h after transfection with sodium butyrate and harvested by centrifugation ~3–5 days post-transfection. Conditioned medium was stored at 2–8 °C until purification. Column chromatography was performed using an Akta Explorer 100 chromatography system at 2–8 °C, with 5 ml of rProtein A-Sepharose packed into a 1.6-cm diameter XK 16 column (GE Healthcare). The linear flow rate was 150 cm/h for each experiment. Conditioned medium was 0.2-μm filtered prior to processing (polyethersulfone, 0.2 μm, from Nalgene (Rochester, NY)). The Protein A-Sepharose column was equilibrated in 5 column bed volumes of PBS, pH 7.4, and loaded with conditioned medium. The column was washed to base-line A_{280} (10 column bed volumes) with PBS, pH 7.4, and bound protein was eluted with 10 column bed volumes of 0.1 M sodium citrate, pH 3.0. Eluate was collected into a container containing one-twentieth elution volume of 1 M Tris-Cl, pH 8.0. Eluate was subsequently exchanged into 20 mM HEPES, 150 mM NaCl, 150 mM L-arginine, pH 6.8, by dialysis (dialysis membrane 10,000 Da molecular mass cutoff; Pierce). Formulated IgG was 0.2-μm filtered and stored at 2–8 °C. Concentration and yield were determined by A_{280} (ϵ = 1.4), and purity was determined by SDS-PAGE (4–12% BisTris gels; prestained See Blue SDS-PAGE standards were from Invitrogen).

IgG Production in Nicotiana benthamiana—Heavy and light chain genes of GCI079 were codon-optimized for expression in *Nicotiana benthamiana*, synthesized (GeneArt, AG), and subsequently cloned into plant (TMV and PVX) expression vectors (Icon Genetics, GmbH), followed by transformation into *Agrobacterium tumefaciens* strain ICF320 (18). For transient expression of IgG079P (IgG format of GCI079 produced in plants) *in planta*, we used the "magnifection" procedure (Icon Genetics, Halle (Saale), Germany) as described (19), with minor modifications. The *N. benthamiana* plants used for *Agrobacterium* infection and subsequent antibody production were obtained from Dr. Herta Steinkellner (Universität für Bodenkultur Wien, Vienna, Austria); these plants were modified by RNAi expression to eliminate the expression of the endogenous plant-specific xylosyl- and fucosyltransferase genes (20). Plants grown for 4 weeks in an enclosed growth culture room at 20–23 °C were used for vacuum infiltration. Equal volumes of overnight-grown *Agrobacterium* cultures were mixed in the infiltration buffer (10 mM MES, pH 5.5, and 10 mM $MgSO_4$) resulting in a 1:1000 dilution for each individual culture. The infiltration solution was transferred into a 20-liter custom built (Kentucky Bioprocessing, Owensboro, KY) vacuum chamber. The aerial parts of entire plants were dipped upside down into the bacterial/buffer solution. A vacuum of 0.5 bars was applied for 2 min. After infiltration, plants were returned to the growth room under standard growing conditions. Eight days postinfiltration, the leaf tissue was extracted in a juicer (Green Star, model GS-1000), using 25 ml of chilled extraction buffer (100 mM Tris, 40 mM ascorbic acid, 1 mM EDTA) per 100 g of green leaf tissue. The plant-derived extract was clarified by lowering the pH of the extract to pH 4.8 with 1 M phosphoric acid then readjusting it to pH 7.5 with 2 M Tris base to insolubilize plant debris, followed by centrifugation at 16,000 × *g* for 30 min. The supernatant was transferred and recentrifuged at 16,000 × *g* for an additional 30 min. The clarified extract was filtered through 0.2 μm prior to loading onto a 5-ml HiTrap MabSelect SuRe (GE Healthcare) Protein A column at 2 ml/min. The column then was washed with running buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) and eluted with 0.1 M acetic acid, pH 3.0. The resulting eluate was neutralized to pH 7 using 2 M Tris, pH 8.0, and supplemented with Tween 80 to 0.01%. The mAb solution was then polished via Q filtration (Mustang Acrodisc Q membrane; Pall), aliquoted, and stored at −80 °C until used. All mAb was fully assembled as determined by SDS-PAGE and had less than 5% aggregate as determined by HPLC-SEC.

Human PBMCs and Toxin Neutralization Assay in Vitro—Peripheral blood mononuclear cells were isolated from heparinized blood of healthy human donors by Ficoll gradient centrifugation as described (21). Isolated peripheral blood mononuclear cells were washed twice in PBS, frozen in 20% DMSO in HI-FBS overnight at −80 °C, and stored in liquid nitrogen until further use. For the assay, the cell pellet was resuspended in RPMI 1640 with 5% fetal bovine serum (FBS), and cells were washed and enumerated by trypan blue exclusion

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and adjusted to 2×10^6 cells/ml. 75 μl of this cell suspension (1.5×10^5 cells) with a viability of >95% was added to duplicate wells of 96-well flat bottom plates containing 37.5 μl of semilog diluted (0.02–20 μg/ml) human monoclonal antibodies and 37.5 μl of SEB. Wells containing medium with toxin only were used as controls. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air for 48 h. Cells were centrifuged at $1600 \times g$ for 10 min, culture supernatants were harvested, and IFNγ production was assessed by ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's protocol. Plates were read at 450 nm using the VersaMax plate reader, and data were transferred and analyzed in Microsoft Office Excel 2007. Cells stimulated with toxin in the absence of a neutralizing agent served as positive control; this was considered as 0% IFNγ inhibition. Accordingly, inhibition of IFNγ production in the presence of neutralizing agent was calculated as the difference between positive control and sample. IC_{50} values for the neutralizing agents (human monoclonal antibodies) were determined using a 4-parameter logistic model (equation 205, XLFit version 5.2).

Enzyme-linked Immunosorbent Assay (ELISA) for Detection of Superantigens by Full-length IgGs—96-well plates were coated with 100 ng of SEA, SEB, SEC1 to -3, SED, SEK, TSST-1, SpeA, or SpeC overnight at 4 °C. Nonspecific binding was blocked with Starting Block PBS® (ThermoScientific) for 2 h at room temperature. Different dilutions of IgGs in Starting Block PBS were incubated for 2 h at room temperature. Bound IgG was detected with a goat anti-human IgG-HRP antibody, and plates were read at 650 nm using a Versamax plate reader. Data were analyzed in Softmax using a 4-parameter logistic curve.

Kinetic Analyses—For surface plasmon resonance analysis, a human IgG capture kit (GE Healthcare) was used to immobilize a polyclonal antibody against human IgG onto the surface of a Biacore CM5 chip (GE Healthcare) using an amine-coupling kit with a target capture level of 1000 Resonance units (RU). This orientation eliminates the chance of detecting avidity effects from the bivalent nature of IgG. 50–150 RUs of mAb were captured, and SEB (Toxin Tech) was flowed over the chip at five different concentrations (with the highest concentration having an R_{max} between 30 and 80 RUs), and kinetic analyses using BIAE valuation software were performed (1:1 fit). Fast flow rates and controls (including a flow cell with no captured human mAb and a run with no SEB) were performed to ensure against acquiring mass transfer-limited data.

Animals—Eight-week-old female BALB/c mice were purchased from Charles River (Willmington, MA). Mice were maintained under pathogen-free conditions and fed laboratory chow and water *ad libitum*. All mouse work was conducted in accordance with protocols approved by institutional animal care and use committees.

Animal Efficacy Studies—For prophylactic protection studies in BALB/c mice, 10 LD_{50} (2 μg/mouse) of SEB was incubated with 90 μg of human monoclonal Fab antibodies for 1 h at room temperature, before intraperitoneal administration in a total volume of 200 μl. Four hours postinjection, SEB toxicity was potentiated with 40 μg of LPS in a 200-μl volume administered intraperitoneally. Time course studies were performed to evaluate the therapeutic activity of full-length human monoclonal antibodies. Mice were challenged with different doses (4, 3, 2.5, 2, 1.5, 1, 0.5, 0.25, and 0.125 μg) of SEB at $t = 0$ h and received 40 μg of LPS at $t = 4$ h via the intraperitoneal route, each in a volume of 200 μl. At $t = 1$ h, mice were treated with 200 μg of either human monoclonal antibodies or nonspecific mouse IgG in a 100-μl volume of PBS. Mice were monitored for morbidity (weight loss, hunched posture, lethargy, ruffled fur) and mortality over a time period of 4 days. For cytokine analysis, mice were challenged with 4 μg of SEB ($t = 0$ h) and received 200 μg of human monoclonal Abs or mIgG 1 h later ($t = 1$ h) and 40 μg of LPS 4 h later ($t = 4$ h). At $t = 5$ h and $t = 8$ h, mice were bled by cardiac puncture, and serum was used to determine proinflammatory cytokines.

SDS-PAGE and Western Blotting—The purity of the antibodies was verified by SDS-PAGE. Each sample (100 ng) was loaded on a 4–15% polyacrylamide gel in SDS loading buffer (Boston BioProducts) and electrophoresed at 140 V for 45 min. The gel was stained with Gelcode Blue Stain Reagent (Thermo Scientific).

The binding properties of the antibodies were determined by Western blotting. SEB (100 ng) was electrophoresed in a 4–15% polyacrylamide gel and subsequently trans-blotted onto a nitrocellulose membrane (0.45 μm; Millipore). The blotting was performed in 1× transfer buffer (NuPage transfer buffer, Invitrogen) at 20 V for 40 min. After blotting, the membrane was blocked by incubation in Starting Block T20 (Invitrogen) for 10 min at room temperature. The blocked membrane was incubated with the antibody sample diluted in Starting Block (1:1000, v/v) overnight. The membrane was washed with 1× TBT-T and incubated with goat anti-human IgG alkaline phosphatase conjugate (Bio-Rad) diluted in Starting Block (1:3000, v/v) for 1 h on a shaker. After three washes with TBS-T, the substrate solution (AP substrate conjugate kit, Bio-Rad) was added, and the band was developed for 10 min, after which the membrane was washed with deionized water and dried on Whatman paper.

Cytokine Analysis—Cytokine levels in serum were assayed using the Milliplex® MAP Mouse Cytokine/Chemokine Polystyrene Bead Panel (catalog no. MPXMCYTO-70K, Millipore Corp. (Billerica, MA)). The protocol established by the company was used. Briefly, mouse serum samples were thawed, mixed by vortexing, and then clarified through filter spin columns (catalog no. UFC30DV00, Millipore Corp.) by spinning at $12,000 \times g$ for 4 min at room temperature. Each standard, control, or undiluted sample, in 25 μl, was added in duplicate to antibody-conjugated beads and incubated in a 96-well filter plate overnight at 2–8 °C with shaking at 650 rpm. After 16–18 h, wells were washed, and 25 μl of detection antibody was added to each well. After 1 h of incubating at room temperature with shaking, 25 μl of streptavidin-phycoerythrin were added to each well and incubated for 30 min with shaking. Final washes were completed, and then 150 μl of sheath fluid was added to each well. The plate was analyzed using a Bio-Plex® 200 suspension array system (Bio-Rad). The instrument settings were as follows: 50 events/bead, 100-μl sample size, and gate settings at 8000–15,000. The software used to perform the assay and analyze data was Bio-Plex Manager™ version 6.0,

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which calculated concentrations in pg/ml based on the respective standard curve for each cytokine.

Statistical Analysis—Data were analyzed using PRISM software (GraphPad Software, Inc.). *In vivo* survival curves were analyzed using the log-rank (Mantel Cox) test.

RESULTS

Selection of Anti-SEB Fabs Using Phage Display—We used phage display technology to select Fabs specifically targeting SEB. The synthetic antibody library was constructed on a single human framework as described previously (22) but with greater diversity allowed in the third hypervariable loop of the light chain.[5] The naive library pool contained $>10^{10}$ unique clones.

The library was cycled through five rounds of binding selections with immobilized SEB antigen. Enrichment was observed from the third round of biopanning. Ninety-six clones from each of rounds 3–5 were tested by single-point competition phage ELISA. Seventy-five clones showed both positive signal in spot phage ELISA and over 50% inhibition in the presence of 10 nM solution phase SEB in single-point competitive phage ELISA. These clones were sequenced, and 20 unique clones were obtained. Sequence analysis indicated that 15 of 20 clones retained the original heavy chain sequences as the template used for library construction, with the other five clones containing 1–3 point mutations in only one of the three heavy chain CDRs. The high occurrence of parental heavy chain CDRs is not due to bias introduced during the construction of the library because sequencing analysis showed that ~70% of the clones in the naive library are fully mutagenized in all four CDRs subjected to mutagenesis, meaning that only 30% of clones contain one or several loop sequences, as found in the parental clone.[5] This correlates well with the typical >80% mutagenesis efficiencies obtained with the mutagenesis procedure used to introduce diversity (23). Moreover, Fab clones containing highly diversified sequences in four CDRs, although displaying high antigen-binding affinity and specificity, were also obtained from the selection using this naive library against different antigens.[6] In contrast, all SEB-binding clones had CDR-L3 sequences different from that in the template, of which 16 clones share the following consensus CDR-L3 sequence: (S/V/A)(Y/W)S(A/S)(S/H/Y/V)(S/Y/H)PF. Therefore, our affinity maturation strategy for further improvement was focused on the consensus sequence of CDR-L3.

First Affinity Maturation—Two sublibraries were constructed for the first round of affinity maturation. In one sublibrary, mutations were introduced into CDR-L3 only. In the other sublibrary, mutations were introduced into both CDR-L3 and one of the other four CDRs that are spatially close to CDR-L3, namely CDR-L1 and the three heavy chain CDRs. These two sublibraries were cycled through five rounds of binding selections with immobilized SEB antigen under more stringent conditions by successively lowering the concentration of immobilized antigen and more intensive washing. We sequenced only those clones that bound to immobilized SEB in spot phage ELISA and exhibited over 50% inhibition in the presence of 5 nM solution-phase SEB in single-point competition phage ELISA. Twenty-two unique clones were obtained from affinity maturation. Interestingly, all of the improved clones had new CDR-L3 sequences, but most retained the original heavy chain CDR sequences, with CDR-L1 and CDR-H3 being non-mutated in all clones, which was also observed in first generation Fabs. This may indicate that the conformation formed by the parental heavy chain CDR sequences is complementary to the epitope on the antigen that the Fabs interact with, but it could also be due to the design of the template with stop codons embedded only in CDR-L3 and therefore the high occurrence of parental heavy chain CDR sequences.

The selected Fabs were expressed in 55244 *E. coli* cells and purified on an rProtein A column. Endotoxin levels were reduced to below 10 Endotoxin Units (EU)/mg by extensive washing of loaded column with nonpyrogenic PBS buffer supplemented with 0.1% Triton 114 followed by nonpyrogenic PBS buffer. The Fabs were then eluted and subjected to buffer exchange to PBS. SDS-PAGE and endotoxin level measurements were performed for quality control purposes.

The purified Fabs were then tested *in vitro* in a toxin neutralization assay (TNA) using SEB-mediated induction of IFNγ production by human PBMCs as a readout, and the best Fabs emerging from the affinity maturation were identified based on IC_{50} values (concentration for 50% inhibition of SEB activity). Furthermore, the binding affinities were estimated in multipoint competition phage ELISA. The selected Fab clones (Table 1), denoted as GCI064, GCI073, GCI075, and GCI079, all showed low binding IC_{50} values (3–5 nM) and low TNA IC_{50} values (36–132 nM).

We tested the *in vivo* neutralization of SEB by GCI064, GCI073, GCI075, and GCI079 in the LPS potentiation model of toxic shock in BALB/c mice. SEB (2 μg) was preincubated with each Fab (90 μg) for 1 h at room temperature. Mice were then challenged with SEB alone or with SEB pretreated with the Fabs by intraperitoneal injection. After 4 h, mice were injected intraperitoneally with 40 μg of LPS and monitored for 4 days for morbidity and mortality. As shown in Fig. 1, whereas all mice treated with SEB and LPS alone succumbed within 2–3 days, preincubation of SEB with the Fabs resulted in 80% (GCI073) to 100% (GCI064, GCI075, and GCI079) protection from lethal challenge.

Second Affinity Maturation—As the template for the second round of affinity maturation, we chose GCI073, one of the four Fabs that demonstrated the best neutralizing efficacy in an *in vitro* toxin neutralization assay from the first round affinity maturation. Three sublibraries were constructed. In the first and second sublibraries, alanine scanning and homolog scanning were utilized to mutagenize CDRs of GCI073. In the third sublibrary, the oligonucleotides used for alanine scanning and homolog scanning were premixed and utilized in site-directed mutagenesis for library construction. These sublibraries were pooled before they were subjected to panning similar to that in the first round of affinity maturation. Three clones (GCI119, GCI120, and GCI121) were identified with slightly improved IC_{50} values (Table 1).

Generation of Full-length Human Anti-SEB IgG1—The heavy and light chain variable region sequences from GCI064, GCI075, GCI079, GCI0119, GCI0120, and GCI0121 were

[6] S. S. Sidhu, unpublished data.

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TABLE 1

Sequences and IC_{50} values of anti-SEB Fabs

CDR sequences are shown for Fabs isolated from library F (*A*); the first round of affinity maturation, in which the library was constructed based on the consensus sequence of CDR-L3 identified in initial panning from library F (*B*); and the second round of affinity maturation, in which the library was constructed based on clone GCI073 (*C*). The numbering is according to the nomenclature of Kabat *et al.* (38). Residues that are different from the template HP153 (top row) used in constructing library F are shown in white with black background. Dashes indicate gaps in the alignment. IC_{50} values are concentrations of SEB that inhibit 50% of Fab-displayed phage binding to immobilized SEB (*a*) or concentrations of Fab protein that block 50% of INF release in the *in vitro* toxin neutralization assay (*b*). ND, not determined.

	CDR-L1	CDR-L2	CDR-L3	CDR-H1	CDR-H2	CDR-H3		
Clone	28 29 30 31 32	50 51 52 53 54 55 56	91 92 93 94 95 95a 95b 96	29 30 31 32 33 34 35 35a	50 51 52 52a 53 54 55 56 57 58	94 95 96 97 98 99 100 100a 100b 100c 100d 100e 100f 100g 100h 100i 101	IC_{50} (nM)[a]	IC_{50} (ug/ml)[b]
HP153	SVSSA	SASSLYS	SSYSL--I	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD		
(A)								
GCI033	SVSSA	SASSLYS	SYGFPL-F	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	ND	
GCI034	SVSSA	SASSLYS	SYSSVSPF	FSSSSIH-	SIYPSYGYTY	RTVRGSKKPYFSGWAMD	13	
GCI035	SVSSA	SASSLYS	SYSASYPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	5	
GCI036	SVSSA	SASSLYS	SYSAHYPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	10	
GCI037	SVSSA	SASSLYS	SYSASSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	15	
GCI038	SVSSA	SASSLYS	SYGSPL-F	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	12	
GCI039	SVSSA	SASSLYS	SYGHPL-F	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	12	
GCI040	SVSSA	SASSLYS	VYSSYSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	12	
GCI041	SVSSA	SASSLYS	SYSAYYPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	13	
GCI042	SVSSA	SASSLYS	SYSAHSPF	FSSSSIH-	SIYSSYGYTY	RTVRGSKKPYFSGWAMD	10	
GCI043	SVSSA	SASSLYS	SYSSSSPF	IYSSSMH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	13	
GCI044	SVSSA	SASSLYS	VWSAHHPF	FSSSSIH-	SIYPSYGYTY	RTVRGSKKPYFSGWAMD	8	
GCI045	SVSSA	SASSLYS	SYSASSPF	FSSSSIH-	SIYPYYGYTY	RTVRGSKKPYFSGWAMD	5	
GCI046	SVSSA	SASSLYS	VYGHPL-F	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	4	
GCI047	SVSSA	SASSLYS	SYSSYSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	10	
GCI048	SVSSA	SASSLYS	VYSAHSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	8	
GCI049	SVSSA	SASSLYS	AYSAYYPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	5	
GCI050	SVSSA	SASSLYS	VYSASSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	11	
GCI051	SVSSA	SASSLYS	SYSAVYPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	8	
GCI052	SVSSA	SASSLYS	SYSAVSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	8	
(B)								
GCI059	SVSSA	SASSLYS	IYSASAPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	5	
GCI060	SVSSA	SASSLYS	IYSAISPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	3.3	
GCI061	SVSSA	SASSLYS	IYSATQPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	2	
GCI062	SVSSA	SASSLYS	IYSARTPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	3	
GCI063	SVSSA	SASSLYS	QYSAQNPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	3	
GCI064	SVSSA	SASSLYS	SYSAQSPF	FSSSSIH-	SIYPSYGYTY	RTVRGSKKPYFSGWAMD	3.1	6.7
GCI065	SVSSA	SASSLYS	SYSARSPF	FSSSSIH-	SIYPGYSYTY	RTVRGSKKPYFSGWAMD	4	
GCI066	SVSSA	SASSLYS	VYSAQHPF	FSSSSIH-	SIYPYYSYTY	RTVRGSKKPYFSGWAMD	2.2	
GCI067	SVSSA	SASSLYS	VYSARHPF	IGGASIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	2.8	
GCI068	SVSSA	SASSLYS	VYSANSPF	IAAGASMH	SISSSYGYTY	RTVRGSKKPYFSGWAMD	3.2	
GCI069	SVSSA	SASSLYS	AYSARYPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	5.7	
GCI070	SVSSA	SASSLYS	IFAAQMPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	10.1	
GCI071	SVSSA	SASSLYS	SFSAMEPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	9	
GCI072	SVSSA	SASSLYS	AYSGSSPF	FSSSSIH-	SIYPGYSYTY	RTVRGSKKPYFSGWAMD	2.8	
GCI073	SVSSA	SASSLYS	VYTAVHPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	3.6	5.9
GCI074	SVSSA	SASSLYS	VYSANYPF	FSSSSIH-	SIYPSYSYTY	RTVRGSKKPYFSGWAMD	3	
GCI075	SVSSA	SASSLYS	VYSSSSPF	LWYHSTMH	SISSSYGYTY	RTVRGSKKPYFSGWAMD	3.6	3.5
GCI076	SVSSA	SASSLYS	AYSSSSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	2.2	
GCI077	SVSSA	SASSLYS	VYSSTSPF	IHGASMH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	2.2	
GCI078	SVSSA	SASSLYS	VYTSTSPF	ISSGSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	4	
GCI079	SVSSA	SASSLYS	AFSARSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	5	1.9
GCI080	SVSSA	SASSLYS	LYGATSPF	FSSSSIH-	SISSSYGYTY	RTVRGSKKPYFSGWAMD	2	
(C)								
GCI119	SVSSA	SASSLYS	VYTAVHPF	FSSSSIH-	SISSSYGYTY	RTVAGSTTADFAGWAMD	1.6	6.3
GCI120	SISSA	SAAALYS	IFSAVHPY	FSSSSIH-	SVSSSYGFTY	RTIRGAKKPFFAGWAMD	1.3	1.1
GCI121	SVSSA	SASALYA	IYSAVNPY	FSSASIH-	AVASSYGYTY	RTVRASAAAAFAGWAMD	1.3	0.3

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inserted into expression vectors pMAZ-IgL or pMAZ-IgH (17), respectively. pMAZ-IgL and pMAZ-IgH contain expression cassettes of the constant region of human κ light chain or human γ1 heavy chain, respectively. To produce full-length antibodies, the mammalian expression vectors for IgH and IgL were used to transfect HEK-293T cells. After initial optimization in 125-ml culture flasks, the production was scaled up to 5 liters under serum-free conditions in suspension culture. The rIgG was purified using Protein A and formulated in PBS. The antibodies were quality-controlled by SDS-PAGE, Western blot, and ELISA (binding to SEB).

We also explored the possibility of producing anti-SEB antibodies in a transient plant expression system (magnICON) (19).

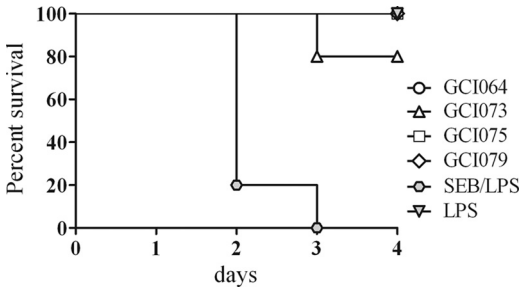

FIGURE 1. ***In vivo* SEB neutralization by Fab clones in mouse model of toxic shock.** BALB/c mice were challenged intraperitoneally with 2 μg of SEB preincubated with Fabs GCI064, GCI073, GCI075, and GCI079 and received 40 μg of LPS 4 h after SEB challenge. Control groups received SEB/LPS or LPS only. Mice treated with GCI064, GCI075, and GCI079 showed full protection ($p = 0.0016$), and those treated GCI073 showed 80% protection ($p = 0.0047$) when compared with SEB/LPS control. Analysis of data was performed using log-rank (Mantel-Cox test) ($n = 5$/group).

By using a transgenic line of *N. benthamiana* lacking plant-specific *N*-glycan residues (20), mAbs with highly homogenous human-like glycoforms can be generated. To this end, the IgG079 (derived from GCI079) heavy and light chains were synthesized and inserted into magnICON expression vectors. These vectors were introduced into *A. tumafaciens*, which was then infiltrated into 1 kg of plants. After 1 week, plants were harvested, and mAb protein was extracted and purified via Protein A chromatography. Approximately 70 mg of IgG079 was recovered. The mAb was quality-controlled by SDS-PAGE (reduced and non-reduced) and SEC-HPLC (data not shown).

Binding of Anti-SEB IgGs to SEB and Other Superantigens—Purified full-length IgGs were first tested in binding ELISA using purified SEB and several other superantigens as coating antigen. As shown in Fig. 2, affinity-matured IgGs (clones 119–121) showed enhanced binding to SEB with about 10-fold improved EC_{50}. Whereas the parental clones showed low binding to SEA and SEC-1, the affinity-matured clones 120 and 121 displayed increased binding to these superantigens (Fig. 2). In contrast, binding to SED observed for the parental clones was reduced upon affinity maturation (Fig. 2).

Determination of Kinetic Properties of Anti-SEB IgGs—Kinetic constants for the three best antibodies were determined using surface plasmon resonance (Biacore) as described under "Experimental Procedures." As shown in Table 2, clones 119, 120, and 121 showed subnanomolar to low nanomolar affinities. Kinetic analyses (Table 2) reveal that the improvements in binding seen with clone 121 are due to both a faster on rate (k_a) and a slower off rate (k_d).

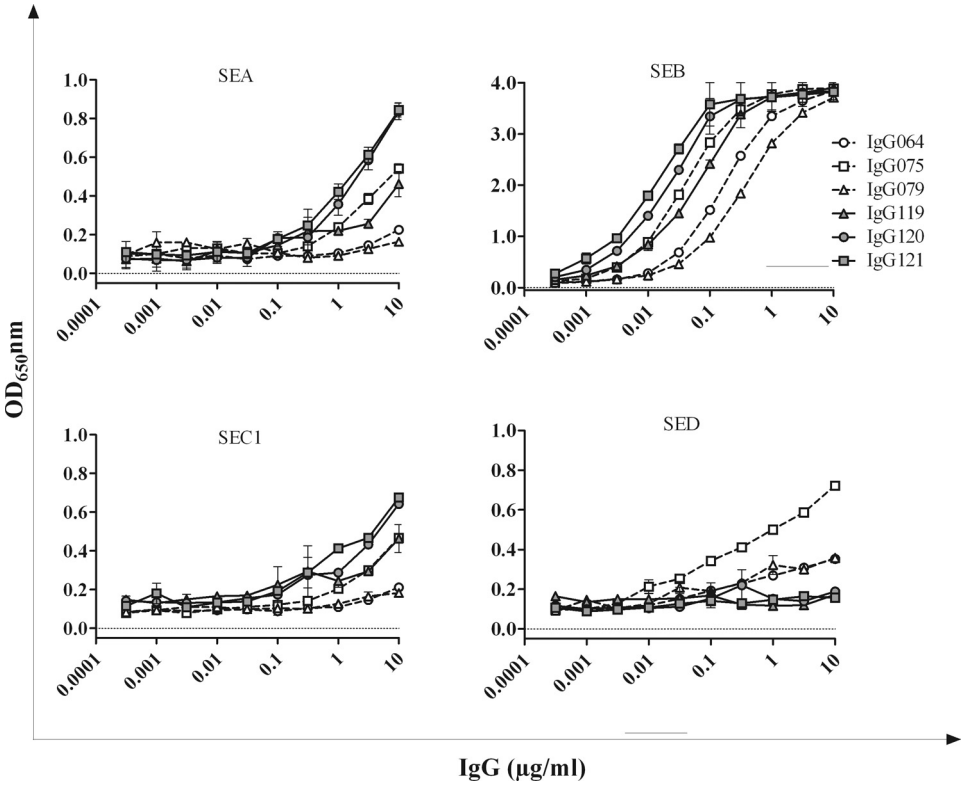

FIGURE 2. **ELISA for detection of superantigens by full-length IgGs.** ELISA was performed with different dilutions of full-length one time affinity-matured IgG064, IgG075, and IgG079 and two times affinity-matured IgG119, IgG120, and IgG121 using plates coated with 100 ng of SEA, SEB, SEC1 to -3, SED, SEK, TSST-1, SpeA, or SpeC. Curves are only shown for antigens where binding was observed: binding of IgG064, IgG075, IgG079, IgG119, IgG120, and IgG121 to SEA, SEB, SEC1, and SED. No binding was observed for SEC2 and -3, SEK, TSST-1, SpeA, and SpeC. *Error bars*, S.E.

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In Vitro Neutralization Activity of Anti-SEB Antibodies—Functional activity of the purified full-length antibodies was tested in a toxin neutralization assay using PBMC from three different donors. In these experiments, SEB was preincubated with eight different concentrations of anti-SEB IgGs before adding to PBMC. After culturing the cells for 48 h, IFNγ was measured in supernatants by ELISA, and the percentage inhibition was calculated based on comparison with control wells without antibody. As shown in Fig. 3 and Table 3, the antibodies from the first affinity maturation (IgG075, IgG079, and IgG079P (where "P" represents "produced in plant *N. benthamiana*")) inhibited SEB activity with IC_{50} values ranging from 1.3 to 25 nM, whereas the activity was markedly enhanced through the second round of affinity maturation, as evident by a left shift in the dose-response curve (Fig. 3) and reduced IC_{50} values ranging from 0.13 to 0.72 nM (Table 3). Importantly, IgG79P produced in *N. benthamiana* showed comparable neutralization activity, suggesting that plant-produced anti-SEB retains the critical biological properties.

TABLE 2

Kinetics of SEB binding by IgG119, IgG120, and IgG121

Surface plasmon resonance (Biacore) was performed using human IgG capture CM5 chips and SEB as the analyte. Values presented are the average of three individual experiments with S.E. *, $p < 0.01$ compared with h-13F6$_{CHO}$ (irrelevant control antibody); **, $p < 0.005$ compared with h-13F6$_{CHO}$.

Clone	Kinetic constant determined by surface plasmon resonance		
	k_a	k_d	K_D
	$\times 10^4$/M s	$\times 10^{-5}$/s	$\times 10^{-9}$ M
GCI119	2.4 ± 0.1	3.7 ± 1.1	1.2 ± 0.4
GCI120	2.3 ± 0.5	2.3 ± 0.7	1.0 ± 0.1
GCI121	4.7 ± 0.4	1.5 ± 0.1	0.32 ± 0.01

Therapeutic Efficacy of Anti-SEB Antibodies in Parenteral Challenge Toxic Shock Model—The therapeutic potential of the antibodies was determined in a series of experiments using the LPS potentiated challenge model (24). Groups of five mice were first challenged intraperitoneally with SEB at different doses (4, 3, 2.5, 2, 1.5, 1, 0.5, 0.25, and 0.125 μg) and were treated with 200 μg of IgG119, -120, or -121 or a nonspecific mouse IgG as control antibody after 1 h. Four hours after SEB challenge, mice were injected intraperitoneally with 40 μg of LPS as a potentiating agent. Mice treated with IgG119 showed full protection at any given SEB challenge dose (Fig. 4*A*). Mice treated with IgG120 (Fig. 4*B*) or IgG121 (Fig. 4*C*) showed 80% protection with single mortalities occurring in each of the groups chal-

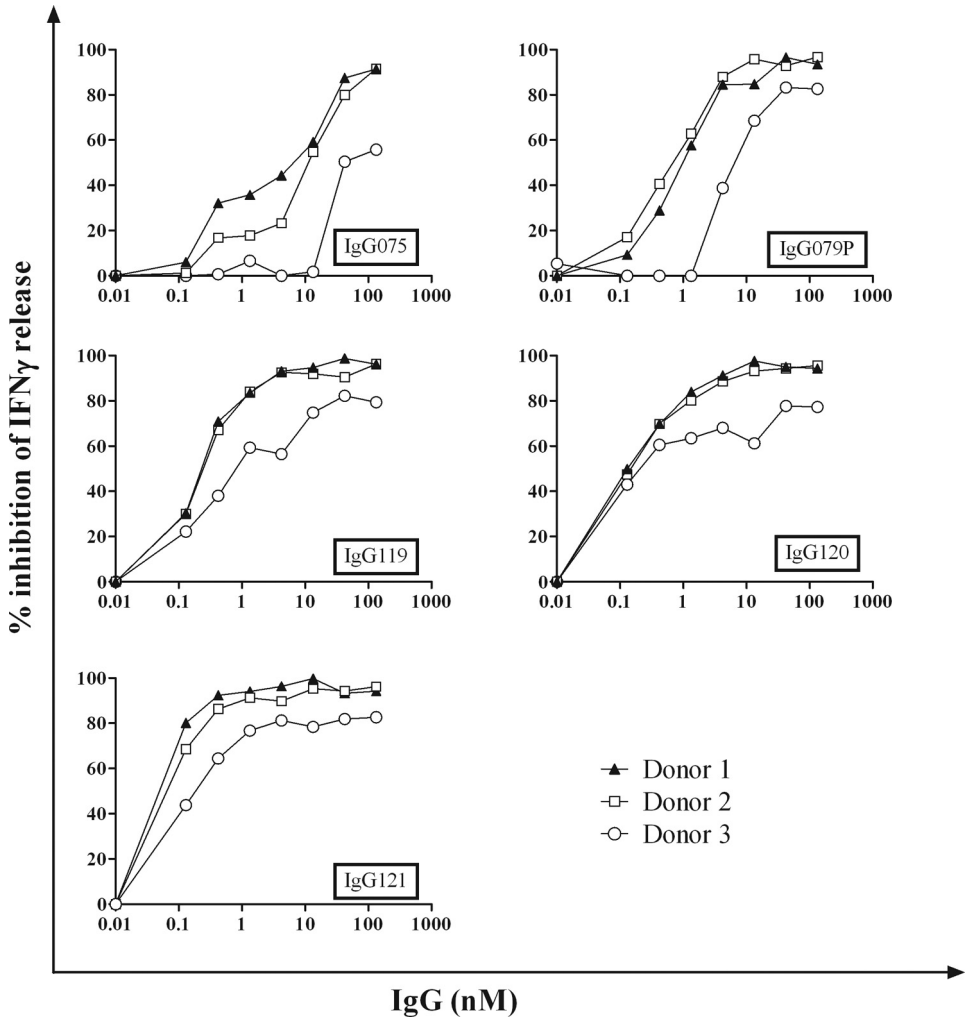

FIGURE 3. ***In vitro* toxin neutralization.** Neutralization of 0.1 ng of SEB by different dilutions of full-length IgGs was performed in human PBMCs. IFNγ was measured by ELISA in the supernatant of stimulated PBMCs as an indicator for T cell proliferation. Shown are TNA curves from duplicates performed with cells from three different donors at different time points.

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TABLE 3
IC_{50} values for anti-SEB IgGs determined in a TNA assay using human PBMC from three donors

Human mAb	Affinity maturation round	TNA IC_{50}				
		Donor 1	Donor 2	Donor 3	Mean	S.D.
				nM		
IgG075	1	3.96	8.89	61.55	24.80	31.92
IgG079	1	0.10	0.15	9.83	3.36	5.60
IgG079-P	1	0.15	0.10	3.56	1.27	1.98
IgG119	2	0.24	0.26	1.65	0.72	0.81
IgG120	2	0.13	0.14	0.15	0.14	0.01
IgG121	2	0.07	0.09	0.25	0.13	0.10

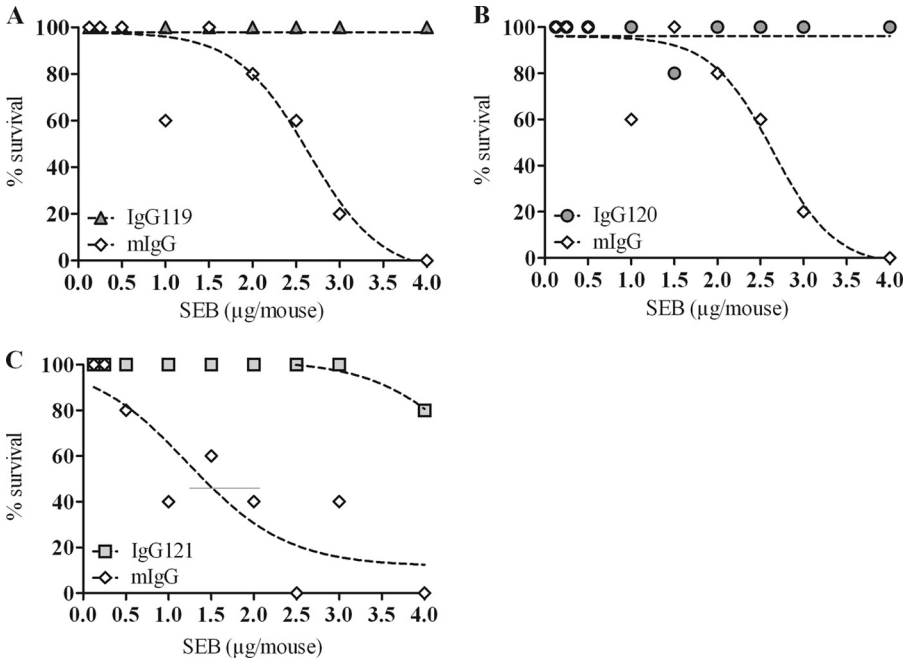

FIGURE 4. **Therapeutic efficacy of human monoclonal IgGs in mouse toxic shock model.** Protection against SEB was tested in BALB/c mice ($n = 5$/group) that were injected with different concentrations of SEB at $t = 0$ h, treated with 200 μg of IgG119 (*gray triangles*, *A*), IgG120 (*gray circles*, *B*), IgG121 (*gray squares*, *C*), or control mouse IgG (*empty squares*, *A–C*) at $t = 1$ h and challenged with 40 μg of LPS at $t = 4$ h. Shown are nonlinear regression curves analyzed in PRISM 5.0.

lenged with 1.5 or 4 μg of SEB, respectively. In groups treated with control mIgG, a clear SEB dose-dependant lethality of animals was observed (Fig. 4, *A–C*). Mice of both treated and mock-treated groups lost up to 10% of their initial weight within 24 h and up to 15% within 48 h after challenge. Most of the mortalities occurred within 28 h but no later than 48 h after SEB challenge. Weight loss during the course of disease was accompanied by ruffled fur, closed inset eyes, and decreased activity. By day 3 postchallenge, weight loss of both treated and mock-treated groups of mice was only 5%, and by day 4 postchallenge, most of the animals had reached their original weight and showed no further signs of morbidity (data not shown).

Inhibition of Cytokine Storm by Anti-SEB Antibodies—To determine the neutralizing effect of the anti-SEB antibodies on the cytokine storm induced by the superantigenic activity of SEB, we determined IFNγ and IL-2 content of serum samples of mice treated with IgG121 or control mIgG. Mice were challenged with SEB ($t = 0$ h) and treated 1 h later ($t = 1$ h) with IgG121 or mIgG and challenged 4 h later with LPS ($t = 4$ h). Five hours ($t = 5$ h) and 8 h ($t = 8$ h) after SEB challenge, 3–5 mice/group were euthanized, and blood samples were collected. Serum was separated, and cytokine levels were determined by a Bioplex assay. IFNγ levels were elevated in mock-treated mice at both time points, with levels being significantly higher at $t = 5$ h than in mice receiving IgG121 ($p = 0.032$; Fig. 5, *A* and *B*). Similarly, IL-2 levels of mock-treated mice were higher, but this difference was only significant at the early time point ($p = 0.036$; Fig. 5, *C* and *D*). These data suggest that the therapeutic administration of the antibodies 1 h after exposure can reduce the cytokine release caused by SEB.

Stability of Anti-SEB Antibodies—We performed a limited accelerated stability study to compare the stability of IgG079 produced in either mammalian cells (IgG079) or in *N. benthamiana* (IgG079P). Antibodies were stored for up to 15 days at −80, 4, 22, 37, or 42 °C. On days 3, 7, 10, and 15, samples were analyzed by SDS-PAGE and for SEB binding by ELISA, and Western analysis was carried out for samples collected on days 6 and 14. TNA was performed with samples collected on day 12, using human PBMCs and inhibition of IFNγ release as a readout. IgG079 and IgG079P both showed remarkable stability up to 15 days, as evidenced by SDS-PAGE analysis (Fig. 6). Stability was also confirmed in the TNA assay, where both IgGs showed very similar neutralizing activity after being incubated under different conditions (Fig. 7). Similar data were obtained for other antibodies tested (data not shown).

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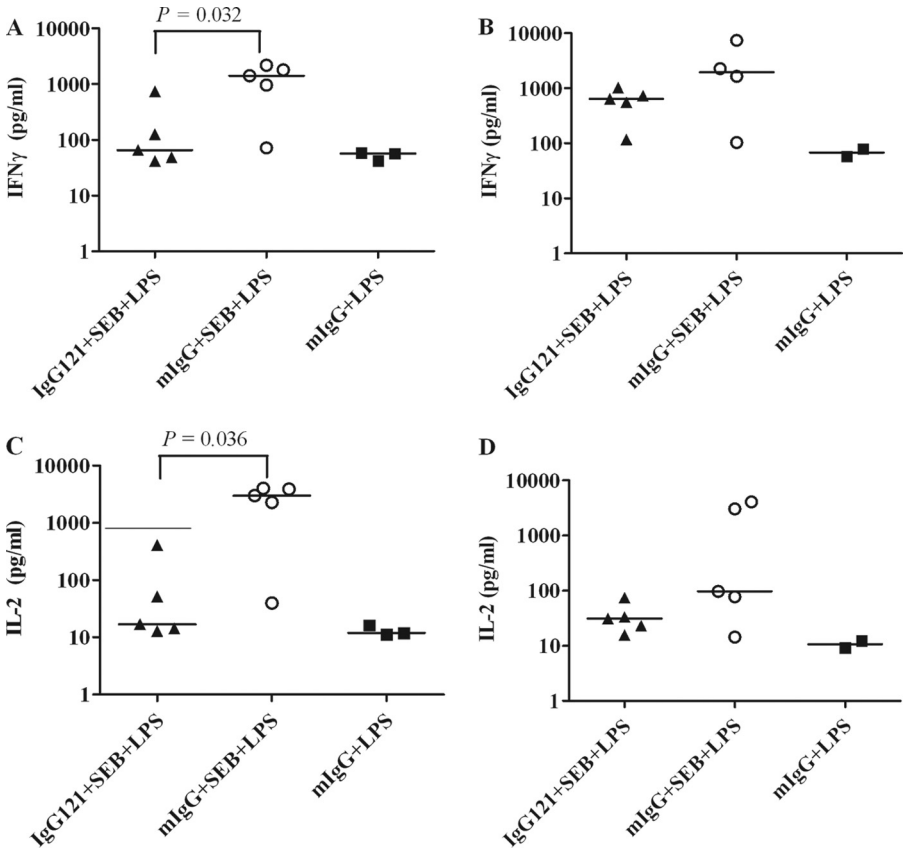

FIGURE 5. **Cytokine production *in vivo*.** IFNγ and IL-2 were assessed in serum samples of BALB/c mice that were challenged with 4 μg of SEB at $t = 0$ h, treated with 200 μg of IgG121 or control mIgG at $t = 1$ h, and challenged with 40 μg of LPS at $t = 4$ h. Serum samples collected at $t = 5$ h (*A* and *C*) and $t = 8$ h (*B* and *D*) were used to determine cytokine levels. Mice receiving control mIgG (*open circles*) demonstrated elevated levels of both cytokines at either time point when compared with mice treated with IgG121 (*closed triangles*) or non-SEB-challenged mice (*closed squares*). Shown are median values of $n = 3$–5 mice/group.

DISCUSSION

Neutralization of virulence factors of bacterial pathogens is a key component of therapeutic and prophylactic strategies to counter the detrimental effects of bacterial infections. Toxin-based vaccination approaches have been used traditionally against several bacterial pathogens, such as *Clostridium tetani*, *Bordetella pertussis*, and *Corynebacterium diphtheriae*. *S. aureus* produces a plethora of toxins aimed at immune evasion and tissue destruction (25). Among these toxins, SAgs, consisting of over 19 toxins, are known for massive induction of a polyclonal T cell activation, resulting in life-threatening TSS (5). SEB, one of the most potent SAgs produced by *S. aureus*, is involved in immune evasion during *S. aureus* infection and is one of the major causative agents of staphylococcal TSS and food poisoning (5, 6, 8). Furthermore, SEB is one of the most potent potential agents of bioterrorism, developed as a bioweapon by the offensive weapons programs of the United States and Soviet Union before the Biological and Toxin Weapons Convention of 1972 (10). Despite this ban, there is serious concern that SEB and other toxins may be used as agents of bioterrorism. It is therefore critical to develop countermeasures to prevent or treat the lethal and incapacitating effects of SEB. Integrated Biotherapeutics is currently developing a vaccine for SEB (STEBVax) for prophylaxis of SEB-induced toxicity (26). However, preparation for an unpredicted or imminent dissemination of SEB would also require a rapidly acting therapeutic.

The success of the toxin-based vaccines is primarily based on antibody-mediated neutralization, an effect that can be replicated in therapeutic settings using animal sera or hyperimmune globulin. However, polyclonal immunoglobulin preparations, while effective, are complicated by the requirement of donor immunization, the requirement of a high dose, and batch to batch variation. Human or humanized monoclonal antibodies represent a safe and effective alternative to the traditional polyclonal approaches.

In this study, we used a methodical approach using screening of a fully synthetic phage display library of human antibodies to identify highly effective neutralizing antibodies against SEB. The lead antibodies were shown to be effective in a postexposure setting against TSS in mice. Furthermore, we have demonstrated the feasibility of production of such antibodies using a novel plant expression system at low costs and high yields, an attribute critically important for a biodefense countermeasure.

By using the highly diverse phage-displayed synthetic antibody library F,[5] we identified a group of Fabs that target SEB with high affinity. Competitive phage ELISA indicated that these antibodies recognized the same epitope on SEB (data not shown). It is not surprising because, except for CDR-L3, 15 of the 20 antibodies share the same CDR sequences in the heavy and light chains as those in the template used for library construction, with the remaining containing only 1–3 mutations in CDR-H1 or CDR-H2 (Table 1). Moreover, the CDR-L3 sequences of these antibodies share significant homology. This

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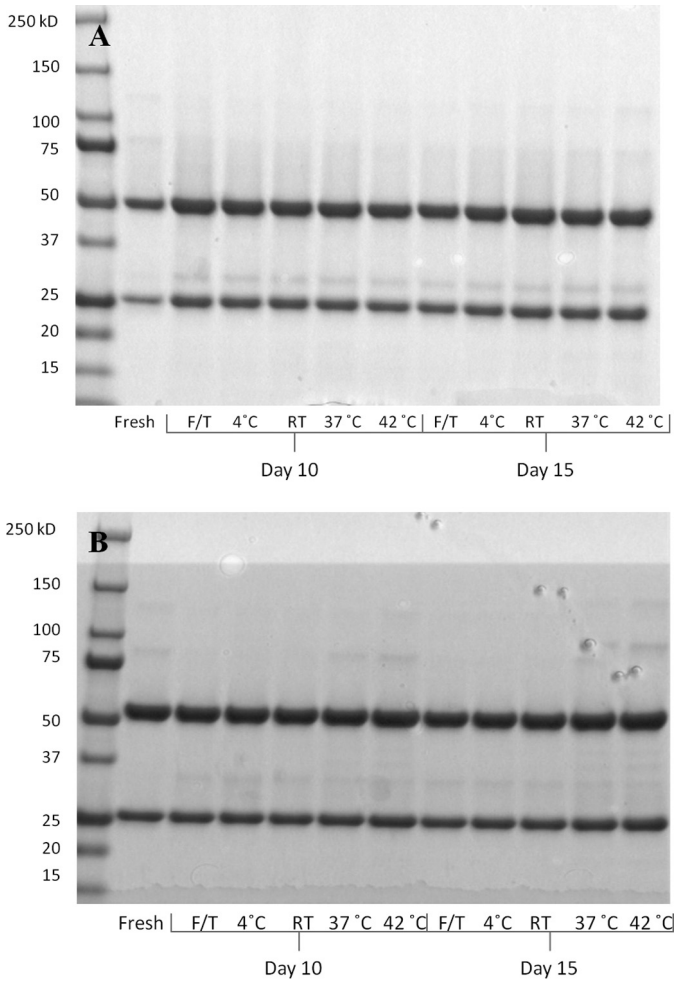

FIGURE 6. **Stability analysis of IgG079 and IgG079P by SDS-PAGE.** SDS-PAGE of antibodies was performed under reducing conditions. Shown are SDS-PAGE profiles of IgG079 (*A*) or IgG079P (*B*) samples that were collected on days 10 and 15 from samples stored at −80 °C (*Fresh*), 4 °C, room temperature (*RT*), 37 °C, or 42 °C or had undergone one freeze-thaw cycle (*F/T*).

indicates that, unlike the traditional view of heavy chain CDRs as major contributors in antigen recognition (27–31) for this group of antibodies, CDR-L3 plays the dominant role in SEB binding. Therefore, the strategy for first round affinity maturation was focused on CDR-L3. By carefully controlling selection conditions, this round of affinity maturation resulted in antibodies with single-digit nanomolar binding affinity, a modest improvement compared with their parental counterparts. In order to further improve antibody affinity, alanine-scanning and homolog-scanning strategies were applied to one of the four best neutralizing antibodies identified in the first round of affinity maturation. Alanine-scanning and homolog-scanning combinatorial mutagenesis are commonly used to determine the functional contributions of individual side chains involved in protein-protein interactions, especially in case where the high resolution structure of the complex is not available (32). We applied these strategies in the hope that by introducing subtle mutations in CDR sequences of our antibody, the existing interactions at the antigen-antibody interface could be fine tuned such that their binding affinity could be improved. This round of affinity maturation further optimized the interaction and resulted in antibodies with subnanomolar affinities for SEB (Table 1).

Thus, the methodical, stepwise approach through *in vitro* affinity maturation used in this study resulted in discovery of highly specific and effective antibodies. Whereas the identified antibodies bind SEB with high affinities, cross-reactivity was also observed to SEA, SEC-1, and SED. The antibodies were shown to inhibit SEB-induced IFNγ secretion by human PBMC as an indicator of superantigenicity of SEB. Neutralization capacity of the antibodies was directly correlated with their affinity, underscoring the importance of identifying high affinity antibodies for effective treatment of SEB-induced toxicity. When used prophylactically or 1 h after exposure, the human antibodies protected mice from lethal challenge with SEB. Previous studies using human anti-SEB antibodies reported by other investigators showed partial protection even when used in a prophylactic setting (33). In another report by Drozdowski *et al.* (34), a human anti-SEB antibody protected 90% of mice when administered at the time of challenge with SEB, whereas the protection dropped to 50 or 10% when antibody was administered 30 min or 1 hour postexposure, respectively. In contrast, three of the lead human anti-SEB antibodies reported here showed complete protection over a wide range of challenge doses when administered 1 h postchallenge.

Protective efficacy of IgG121 was also reflected in the cytokine levels induced *in vivo*. SAg-induced T-cell activation leads to massive systemic release of proinflammatory cytokines and can result in toxic shock (35). SAgs activate $CD4^+$ T cells, primarily inducing Th1 cytokines like IFNγ and IL-2 but only minimal amounts of Th2 cytokines like IL-4 and IL-5. In this manner, SAgs contribute to suppression of a protective humoral response capable of neutralizing staphylococcal toxins. We showed that mice treated with IgG121 had significantly lower levels of both cytokines IFNγ and IL-2 in serum samples 5 h after SEB challenge than mice that were mock-treated.

An important aspect of the current study is the demonstration of the feasibility of producing anti-SEB human antibodies in a plant expression system. As compared with conventional mammal-based expression systems, expression in *Nicotiana* with virus-based transient expression vectors results in the production of extremely high amounts of mAbs in a matter of days (36). Second, the availability of transgenic plants with altered glycosylation pathways enables the production of mAbs with mammalian glycoforms (20). Proteins, including mAbs, have been produced with this system under good manufacturing practice and tested clinically (18, 37). Thus, development of therapeutics against biothreat agents, such as SEB, via this plant-based method offers a rapid, versatile, low cost, and large capacity system to rapidly address a bioterror event.

Although prophylactic use of human antibodies in case of an imminent threat of SEB intoxication in a biowarfare scenario is feasible, the ability to use such agents in a postexposure therapeutic setting is highly desirable from logistic and economic perspectives. The current study underscores the critical importance of identifying high affinity antibodies during discovery for a successful postexposure therapeutic against SEB. Toxins such as SEB act very quickly and thus leave a very short window of opportunity for postexposure intervention. The only way to

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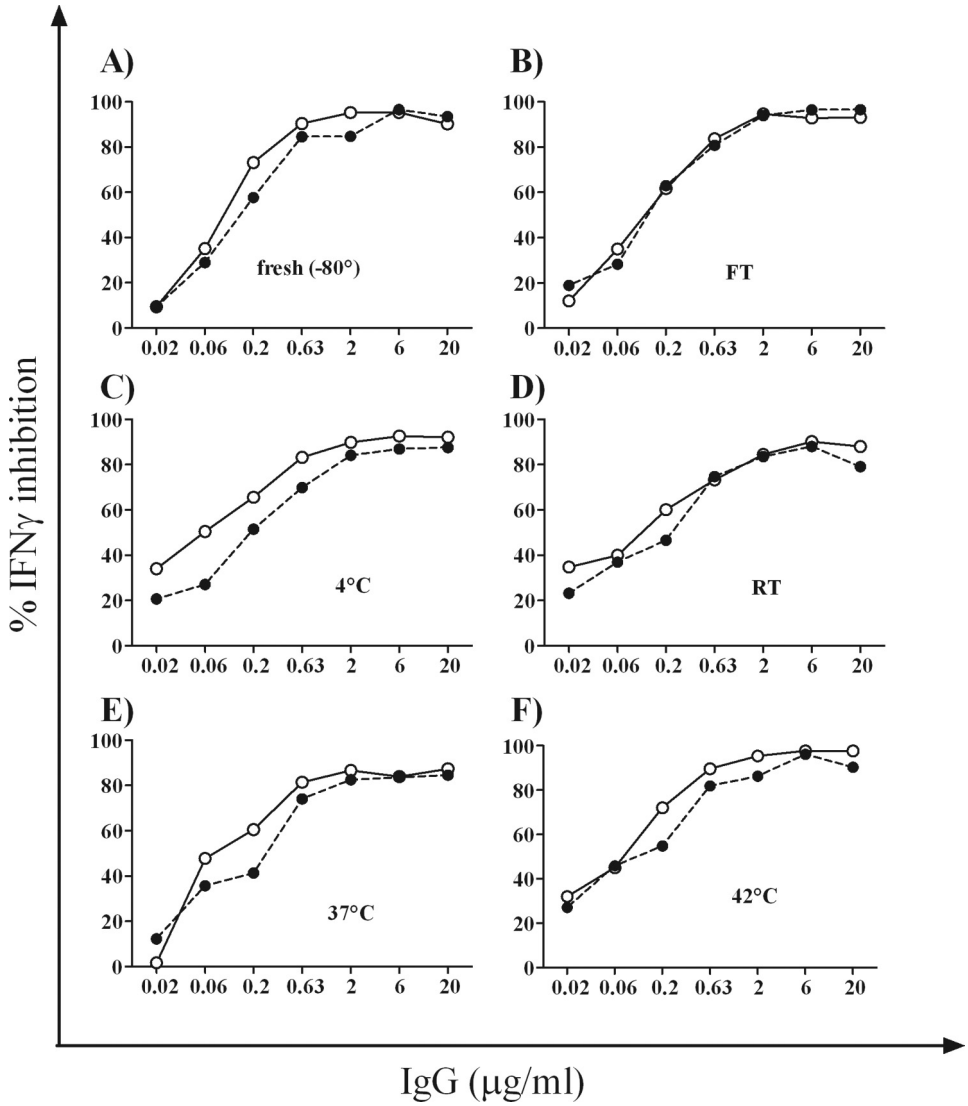

FIGURE 7. **Stability analysis of IgG079 and IgG079P by TNA.** Toxin-neutralizing activity of IgG079 (*open circles*) was compared with neutralizing activity of IgG079P (*closed circles*). Samples were collected after storage under different conditions on day 15 and were tested for their neutralizing activity in TNA using human PBMCs from two different donors. *RT*, room temperature; *FT*, freeze-thaw.

improve the chances of therapeutic success is to develop high affinity and highly specific antibodies with a high on-rate that could effectively and rapidly neutralize the majority of the circulating toxin molecules. Although the reported lead candidates already show a postexposure therapeutic profile, further improvement of this attribute of the antibodies would be highly desirable.

Despite a clear protective effect, treatment with anti-SEB antibodies did not result in reduction of weight loss or obvious signs of morbidity in surviving animals. However, a clear effect was observed on cytokine release, a hallmark of toxic shock. Future studies should further focus on improving the antibodies for significant reduction of symptoms after onset.

To this end, we are currently exploring the development of bispecific antibodies that target two distinct epitopes on SEB. Bispecific antibodies can immensely increase the neutralization capacity of the antibodies. Furthermore, combination treatments using antibodies targeted against different epitopes could be pursued to improve therapeutic efficacy. In addition, these antibodies need to be tested in the nonhuman primate model of SEB pulmonary intoxication. We are currently in the process of scaling up the production process for these antibodies in plants to produce sufficient material for nonhuman primate studies. If successful in nonhuman primate studies, these antibodies represent excellent lead candidates for clinical development.

Acknowledgments—We thank Danielle Carranza for technical assistance and Itai Benhar and George Georgiou for kindly providing IgG expression vectors.

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Supplementary Information

Oligonucleotides

Oligonucleotides containing trimer phosphoramidites were synthesized by TriLink BioTechnologies, Inc. (San Diego, CA). A custom trimer phosphoramidite mixture (Glen Research, Sterling, VA, USA) designated X contains codons for 9 amino acid residues in the following molar ratios: Tyr (25%), Ser (20%), Gly (20%), Ala (10%) and 5 other natural amino acid residues Phe, Trp, His, Pro and Val (5% each). Another trimer phosphoramidite mix designated Z contains codons for 3 amino acid residues in the following molar ratios: Tyr (50%), Ser (25%), Gly (25%). The other oligonucleotides were synthesized with standard methods by Integrated DNA technologies (San Diego, CA).

Other than trimer phosphoramidites, DNA degeneracies are represented in the IUB code (K = G/T, M = A/C, D = A/ G/T, R = A/G, S = G/C, W = A/T, Y = C/T, N = A/T/G/C). Degenerate codons are shown in bold text.

Table S1. Oligonucleotides used in this study

Name	Sequence (5'-3')	Application
L1-5	ACCTGCCGTGCCAGTCAG**XXXXX**GTAGCCTGGTATCAACAG	Mutagenesis in CDR-L1
L1-6	ACCTGCCGTGCCAGTCAG**XXXXXX**GTAGCCTGGTATCAACAG	Mutagenesis in CDR-L1
L3-a	ACTTATTACTGTCAGCAA**NNKTDKRSTRSTNNKNNK**CCGTTCACGTTCGGACAGGGTACC	Mutagenesis in CDR-L3
L3-b	ACTTATTACTGTCAGCAA**KYT**TAT**RSTRSTNNKYMY**CCGTTCACGTTCGGACAGGGTACC	Mutagenesis in CDR-L3
H1-4	GCAGCTTCTGGCTTCAAC**MTCXXXXATS**CACTGGGTGCGTCAG	Mutagenesis in CDR-H1
H1-5	GCAGCTTCTGGCTTCAAC**MTCXXXXXATS**CACTGGGTGCGTCAG	Mutagenesis in CDR-H1
YSG-CDRH2-6	GCCTGGAATGGGTTGCA**Z**ATT**ZYCTZZRGCZ**ACT**Z**TATGCCGATAGCGTC	Mutagenesis in CDR-H2
H3-13	CGTCTATTATTGTGCTCGC**XXXXXXXXXXXXXXGSTWTK**GACTACTGGGGTCAAG	Mutagenesis in CDR-H3
073-L1-A	ACCTGCCGTGCCAGTCAG**KCCGYTKCCKCCGST**GTAGCCTGGTATCAACAG	Alanine scan in GCI073 CDR-L1
073-L2-A	CCGAAGCTTCTGATTTAC**KCCGSTKCCKCCSYTKMTKCC**GGAGTCCCTTCTCGCTTC	Alanine scan in GCI073 CDR-L2
073-L3-A	ACTTATTACTGTCAGCAA**GYTKMTRCTGSTGYTSMTSCAKYT**ACGTTCGGACAGGGTACC	Alanine scan in GCI073 CDR-L3
073-H1-A	GCAGCTTCTGGCTTCAAC**KYTKCCKCCKCCKCCRYT**CACTGGGTGCGTCAGGCC	Alanine scan in GCI073 CDR-H1
073-H2-A	GGCCTGGAATGGGTTGCA**KCCRYTKCCKCCKCCKMTGSTKMTRCTKMT**TATGCCGATAGCGTCAAG	Alanine scan in GCI073 CDR-H2
073-H3-A	GTCTATTATTGTGCTCGC**RCTGYTSSTGSTKCCRMARMASCAKMTKYTKCCGSTKSGGSTRYG**GACTACTGGGGTCAAGGA	Alanine scan in GCI073 CDR-H2
073-L1-H	ACCTGCCGTGCCAGTCAG**KCCRTTKCCKCCKCT**GTAGCCTGGTATCAACAG	Homolog scan in GCI073 CDR-L1
073-L2-H	CCGAAGCTTCTGATTTAC**KCCKCTKCCKCCMTCTWCKCC**GGAGTCCCTTCTCGCTTC	Homolog scan in GCI073 CDR-L2
073-L3-H	ACTTATTACTGTCAGCAA**RTTTWCASCKCTRTTMACSCATWC**ACGTTCGGACAGGGTACC	Homolog scan in GCI073 CDR-L3
073-H1-H	GCAGCTTCTGGCTTCAAC**TWCKCCKCCKCCKCCRTT**CACTGGGTGCGTCAGGCC	Homolog scan in GCI073 CDR-H1
073-H2-H	GGCCTGGAATGGGTTGCA**KCCRTTKCCKCCKCCTWCGSTTWCASCTWC**TATGCCGATAGCGTCAAG	Homolog scan in GCI073 CDR-H2
073-H3-H	GTCTATTATTGTGCTCGC**ASCRTTARGGSTKCCARGARGSCATWCTWCKCCGSTTKGKCTMTG**GACTACTGGGGTCAAGGA	Homolog scan in GCI073 CDR-H3
IgG-VL-BssHII	CAGGCGCGCACTCCGATATCCAGATGACCCAG	Forward primer for VL amplification in IgG construction
IgG-VL-BsiWI	CACCGTACGTTTGATCTCCACCTTGGTAC	Reverse primers for VL amplification in IgG construction
IgG-VH-BssHII	CAGGCGCGCACTCCGAGGTTCAGCTGGTGGAGTC	Forward primer for VH amplification for IgG construction
IgG-VH-NheI	GGTGCTAGCCGAGGAGACGGTGACC	Reverse primer for VH amplification for IgG construction

X = Y/S/G/A/F/W/H/P/V (25/20/20/10/5/5/5/5/5); Z=Y/S/G (50/25/25)

X = Y/S/G/A/F/W/H/P/V (25/20/20/10/5/5/5/5/5); Z=Y/S/G (50/25/25