Effective Treatment of Staphylococcal Enterotoxin B Aerosol Intoxication in Rhesus Macaques by Using Two Parenterally Administered High-Affinity Monoclonal Antibodies

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ABSTRACT Staphylococcal enterotoxin B (SEB) is a protein exotoxin found on the cell surface of Staphylococcus aureus that is the source for multiple pathologies in humans. When purified and concentrated in aerosol form, SEB can cause an acute and often fatal intoxication and thus is considered a biological threat agent. There are currently no vaccines or treatments approved for human use. Studies with rodent models of SEB intoxication show that antibody therapy may be a promising treatment strategy; however, many have used antibodies only prophylactically or well before any clinical signs of intoxication are apparent. We assessed and compared the protective efficacies of two monoclonal antibodies, Ig121 and c19F1, when administered after aerosol exposure in a uniformly lethal nonhuman primate model of SEB intoxication. Rhesus macaques were challenged using small-particle aerosols of SEB and then were infused intravenously with a single dose of either Ig121 or c19F1 (10 mg/kg of body weight) at either 0.5, 2, or 4 h postexposure. Onset of clinical signs and hematological and cytokine response in untreated controls confirmed the acute onset and potency of the toxin used in the challenge. All animals administered either Ig121 or c19F1 survived SEB challenge, whereas the untreated controls succumbed to SEB intoxication 30 to 48 h postexposure. These results represent the successful therapeutic in vivo protection by two investigational drugs against SEB in a severe nonhuman primate disease model and punctuate the therapeutic value of monoclonal antibodies when faced with treatment options for SEB-induced toxicity in a postexposure setting.

KEYWORDS Macaca mulatta, aerosol, antibody therapy, staphylococcal enterotoxin

The staphylococcal enterotoxins (SEs) are a well-characterized family of proteins secreted by Staphylococcus aureus that are known to be toxic at very low concentrations (1). Staphylococcal enterotoxin B (SEB) is a member of the family of superantigens (SAgs), which are microbial proteins that induce polyclonal T-cell activation, in contrast to conventional antigens that undergo proteolytic processing by antigen-presenting cells (APCs) and are presented as a major histocompatibility complex (MHC)/peptide complex (2–4). SAgs bypass these specific mechanisms of antigen presentation by binding outside the peptide binding groove of MHC class II (MHC-II) on APCs and the variable region of T-cell receptor (TCR) β chain on T cells (5–7). Cross-linking of MHC-II and TCR by SAgs activates both APCs and T cells. SAg binding activates 5 to 20% of

Citation Verreault D, Ennis J, Whaley K, Killeen SZ, Karauzum H, Aman MJ, Holtsberg R, Doyle-Meyers L, Didier PJ, Zeitlin L, Roy CJ. 2019. Effective treatment of staphylococcal enterotoxin B aerosol intoxication in rhesus macaques by using two parenterally administered high-affinity monoclonal antibodies. Antimicrob Agents Chemother 63:e02049-18. https://doi.org/10.1128/AAC.02049-18.

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Received 25 September 2018 Returned for modification 23 December 2018 Accepted 13 September 2019 Accepted manuscript posted online 19 February 2019 Published 25 April 2019

May 2019 Volume 63 Issue 5 e02049-18 Antimicrobial Agents and Chemotherapy aac.asm.org
circulating T cells bearing specific V beta regions, leading to massive release of proinflammatory cytokines, activation of cell adhesion molecules, increased T-cell proliferation, and eventual T-cell apoptosis/anergy (8). This sequence of events can culminate in a life-threatening condition clinically referred to as toxic shock syndrome (TSS), marked by cytokine storm, rash, hypotension, fever, multisystem dysfunction, and death (9).

SEB is a prototype SAg with a potential to be used as an airborne, foodborne, or waterborne toxic agent and therefore classified by the CDC as a select agent and by the U.S. National Institutes of Health as a category B priority pathogen. It was developed as a bioweapon in the 20th century due to its incapacitating or lethal nature at much a lower dose than required by many chemical agents. SEB has been considered a high-risk toxin because of its relative ease of production, temperature-independent stability, and exquisite toxicity by the inhalation route. Inhalation of SEB aerosols far exceeds other modalities of exposure in terms of potency and deleterious effects, all initiating at a remarkably low (inhaled) dose. When inhaled, nanogram levels of SEB are incapacitating in humans (half-maximal effective dose \( ED_{50} = 0.0004 \, \mu g/kg \) of body weight), while microgram doses of SEB can be lethal (half-maximal lethal dose \( LD_{50} = 0.02 \, \mu g/kg \)) (1). Inhaled SEB initiates a nearly instantaneous response in the lungs after inhalation, marked by neutrophilic influx, massive cytokine release, and marked pathological changes (10–14). Major osmotic shifts in the lung tissue from SEB inhalation result in a primarily localized inflammatory response which leads to progressive vascular leak, microcapillary hemorrhage, and alveolar flooding (10, 15, 16). The use of animal models of SEB intoxication to evaluate potential treatments is complicated by decreasing sensitivity based upon phylogenetic evolution; murine species are generally unresponsive to SEB unless genetically manipulated (17) or the reaction is potentiated by coadministration of an agent such as lipopolysaccharide (LPS) (15, 18). Nonhuman primate species have been shown to be the closest disease model to study pathophysiology of SEB-induced toxicity or in the testing of promising therapies and vaccine products (11, 19–22).

There are currently no vaccines or therapies approved by the U.S. Food and Drug Administration for either preventing or treating SEB intoxication by any modality of exposure. To date the development of a vaccine has been decidedly slow (23), although research has progressed on the development of STEBVax (24). Research and development on possible therapeutic agents have been even less successful. Known anti-inflammatories, such as dexamethasone, have been used with some success when administered prophylactically (25). Experimental treatments such as CD44 ligand, MyD88 mimetic, and interleukin 1 (IL-1) binding products have also shown promise (26–29) as treatment for SEB-induced shock and acute lung injury. Currently, the most promising postexposure treatment for SEB intoxication appears to be from experimental monoclonal antibodies (MAbs) engineered to target binding and effectively neutralizing any further processing of the molecule by host systems (30–33). The monoclonal antibody Ig121 was shown to protect against systemic exposure in a murine potentiation challenge model (34), although this particular effort did not include small-particle aerosol exposure as a modality of challenge. The c19F1 MAb product has shown to be protective in follow-up murine SEB challenge studies in our laboratories, although the product was one of three MAbs administered as a combination product (35).

In this study, we tested two monoclonal antibodies (IgG121 and c19F1) in a severe model of aerosol SEB intoxication in the nonhuman primate. Each MAb targets different areas of the SEB molecule: Ig121 targets the T-cell receptor, whereas c19F1 binds to the variable \( \beta \) binding region of SEB (34). Binding differences between products are demonstrated \textit{in vitro} using either SEB or the STEBVax vaccine product in preliminary experiments. In the animal experiments, we exposed naïve rhesus macaques to a lethal dose of aerosolized SEB and treated them via intravenous (i.v.) infusion at prescribed times after exposure (either 0.5, 2, or 4 h) to test the ability of each MAb product to ameliorate and/or prevent the effects of intoxication.
RESULTS

Binding ELISA. In order to evaluate the differences in potential binding regions between c19F1 and IgG121, enzyme-linked immunosorbent assays (ELISAs) were developed that use SEB or an attenuated form of SEB (STEBVax) as a coating antigen. STEBVax is a recombinant mutated form of SEB containing three point mutations (L45R, Y89A, and Y94A) that disrupt the interaction of the toxin with human MHC class II receptors and render the protein nontoxic while retaining the immunogenicity (36). When these antibodies were tested in the two ELISAs, c19F1 was able to bind to SEB and STEBVax with similar half-maximal effective concentrations (EC50). In contrast, IgG121 was able to bind to SEB but failed to bind to STEBVax at antibody concentrations as high as 1 μg/ml (Fig. 1). These data suggest that IgG121 binds to the MHC binding surface of SEB while c19F1 binds to a distinct neutralizing site.

MAbs IgG121 and c19F1 protection of rhesus macaques after SEB challenge. Rhesus macaques were challenged via the aerosol route with SEB toxin, and the challenge was measured as consistent between sham- and antibody-treated groups (Fig. 2). The candidate MAbs were i.v. administered at a dose of 10 mg/kg at 0.5 h or 4 h (c19F1) or 2 h or 4 h (Ig121) and yielded complete survival, in contrast to zero survival in sham-treated control groups (Fig. 3).

Hematology. Hematological differences between control and treatment groups were minimal. MAb-treated and sham-treated groups showed a dramatic increase of neutrophils postexposure compared to preexposure values (Fig. 4). Neutrophil percentages of values of the survivors in the treatment group were similar to those in the
control group animals. Similarly, lymphocyte changes in both groups were similar in profile; change in preexposure values significantly decreased postexposure. Decreases were also observed in monocyte percentages.

**Clinical chemistries.** There were blood chemistry differences between the control group and treatment group. The average aspartate aminotransferase (AST) was observed to increase in sham-treated control animals compared to prechallenge levels.
Fig. 5, and the early time point MAb interventions for both c19F1 (0.5 h) and IgG121 (2 h) showed no change compared to prechallenge control. The AST levels increased to levels comparable with those of sham-treated controls in the 4-h MAb-treated groups for both c19F1 and IgG121. A notable increase in alanine aminotransferase (ALT) was present in the sham-treated control animals and the MAb-treated animals. Other parameters of note included blood urea nitrogen (BUN) and creatinine increases in all controls and all but the 0.5-h c19F1 (BUN) and 0.5-h c19F1 and 4 h IgG121 (creatinine) groups (Fig. 6). Other notable changes were a significant decrease in serum protein in
the 0.5-h c19F1 treatment group and a reduction in serum albumin in sham control and 4-h c19F1 and 2-h IgG121 treatment groups.

**Cytokines.** Cytokine and chemokines were measured in replicate serum samples in all animals prior to and 24 h and 48 h after intoxication SEB challenge. Analysis was performed using a Bio-Plex 200 suspension array system, allowing analysis for 28 analytes in a single serum sample. Results of this analysis indicated a strong inflammatory systemic response as a consequence of SEB challenge, with discretion of cytokine response between treated and control animals (Fig. 7). Notably lower concentrations were mainly early-phase proinflammatory cytokines and chemokines, including interleukin 1 receptor antagonist (IL-1RA), macrophage migration inhibitory factor (MIF), monokine-induced gamma (MIG), monocyte attractant protein 1 (MCP1), eotaxin (eosinophilic chemoattractant), and interleukin 6 (IL-6) at the early (0.5-h) c19F1 treatment intervention at 24 h postinfection (p.i.) compared to preexposure levels. There was a noticeable distinction between the gradient of concentrations of cytokines and chemokines between time points in the course of response (24 h versus 48 h) in both the treated and control animals.

**Lung pathology.** Lung damage due to SEB aerosol exposure was measured through wet lung weight in all sham and MAb treatment groups. In all cases treatment with either c19F1 or IgG121 resulted in a significant reduction in wet lung weight, with no difference distinguishable among the treatment groups (Fig. 8).
Histopathology. Five of six SEB-exposed sham treatment group animals developed severe pulmonary edema and mild intra-alveolar inflammation dominated by neutrophils, with most demonstrating edema and acute mild inflammation in the bronchial node (Fig. 9). One animal had minimal pulmonary edema without inflammation but acute randomly distributed necrotizing hepatitis and a small abscess in the mesenteric node consistent with bacterial infection. Mild chronic colitis was detected in four of six animals.

SEB-exposed animals treated with IgG121 or c19F1 were nearly indistinguishable, as evidenced by similar categorical lesion scores of lung tissue from both treatment groups in contrast to the sham-treated animals (Fig. 10). Most had minimal to mild lymphoid hyperplasia in the lung and mild to moderate lymphoid hyperplasia of the bronchial node and spleen, and only three in the c19F1 group presented with minimal widely scattered foci of chronic inflammation in the lungs. Edema was not present in lung or bronchial nodes, and only one animal in the c19F1 group had a minimal degree of colitis.

DISCUSSION

The misuse of biological toxins such as SEB remains a threat to both the military and civilian populations. Recent attempts of poisonings through various mechanisms,
including the U.S. postal service (37), are demonstrative of the ever-present threat and corresponding medical need for rapid-acting countermeasures. In this study, we have addressed the possibility of providing efficacious postexposure therapies to prevent the effects associated with SEB intoxication that would be suitable for use in humans after an accidental or intentional misuse exposure to SEB. The results reported are an important demonstration of a protective outcome in a nonhuman primate model of SEB intoxication and pathogenesis using one of two monoclonal antibodies. c19F1 and Ig121 are the only drug candidates demonstrated to be effective postexposure against

FIG 8 Wet lung weights of animals at necropsy. Lines and error bars represent means and SDs of each group. Weights of sham-treated animals were significantly different than all treated groups. *, significance at a $P$ value of $<0.05$. There was no significant difference in lung weight between treatment groups.

FIG 9 Representative tissues from MAb-treated (lgG121 and 19F1 at 10 mg/kg each) and sham controls. Lungs from treated animals showed minimal inflammation; bronchial lymph nodes of treated animals showed mild lymphoid, diffuse hyperplasia. Lungs from sham-treated animals showed severe inflammation and acute fibrinous accumulation; bronchial lymph nodes showed inflammation and edema. The bar represents 100 μm.
aerosolized SEB in the rhesus macaque model, which is uniformly lethal given the appropriate dose and modality of exposure (aerosol).

The animals that survived exposure through the therapeutic administration of either of the MAbs showed a different profile in clinical response than the untreated controls. Hematological and blood chemistry changes showed minimal differences when group responses were compared. Pathological outcomes show clear differences in damage in the control animals that died from exposure compared to treated survivors, which showed minimal changes in the lung as a result of aerosol exposure. Together, the data derived from the efficacy experiments to date indicate that the anti-SEB MAbs c19F1 and IgG121 are the only effective antitoxin therapies that have been identified that could potentially be used to treat intoxication in humans. In order to develop these MAbs as treatments for SEB intoxication, however, further work is needed. Elucidation of the mechanism of action, binding sites, and toxicological studies is needed to ensure that there are no safety concerns. In addition, the prospect of humanizing c19F1 in order to increase protection while reducing unwanted human immunogenicity may be warranted and may further improve the acceptability of this combination treatment as an emergency clinical product.

MATERIALS AND METHODS

Study design. An initial pharmacokinetics study was performed at the Tulane National Primate Research Center (TNPRC) to determine the optimal route of administration for each of the antibodies (data not shown); animals were administered either antibody either intramuscularly (i.m.) or i.v. and bled according to a predetermined schedule. Serum was analyzed via Biacore (L. Zeitlin) for determination of concentration of the antibody. Intravenous infusion was determined to be the optimal route of administration based upon the rapidity (~30 min) which the antibodies reached serum levels consistent with protection. In the therapeutic studies, all animals were first exposed to SEB aerosols at a target dose consistent with lethality. Thereafter, groups were administered either c19F1 or IgG1 via i.v. infusion at equivalent doses (10 mg/kg) at prescribed times (0.5, 2, or 4 h) postexposure. Sham-treated animals were administered sterile water for injection by that same route.

Animals. Age- and sex-matched rhesus macaques (Macaca mulatta) weighing between 4 and 8 kg, free of simian immunodeficiency virus (SIV), simian type D retrovirus, and simian T-lymphotropic virus, were used. All experiments using macaques were approved by the Tulane Institutional Animal Care and Use Committee. The Tulane National Primate Research Center (TNPRC) is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility (AAALAC no. 000594). The U.S. National Institutes of Health (NIH) Office of Laboratory Animal Welfare assurance number for the TNPRC is A3071-01. Nonhuman primate housing consisted of individual open metal caging units that allowed visual recognition and protected contact with other study animals in the room. Animals were maintained on standard primate chow supplemented daily with fresh fruits and vegetables. Animals were provided standard environmental enrichment during this study, which included
manipulable items in cages, perches, foraging/task-oriented feeding methods, and human interactions with caretakers and research staff. All clinical procedures, including administration of anesthesia and anesthetics, were carried out under the direction of a laboratory animal veterinarian. Animals were anesthetized with ketamine hydrochloride for blood collection procedures. All possible measures are taken to minimize discomfort of all the animals used in this study. Animals were closely monitored daily following surgery for any signs of illness such as anorexia, lethargy, diarrhea, vomiting, and dehydration. Appropriate medical care was implemented if any of these signs of illness were noted. If euthanasia was required in the judgment of the TNPRC veterinary staff, animals were euthanized in accordance with the recommendations of the panel on Euthanasia of the American Veterinary Medical Association. The standard method of euthanasia for nonhuman primates at the TNPRC is anesthesia with ketamine and administration of the antibody was based upon prevailing body weights of the animals enrolled in the study. During the time between the extraction and use of the antibody, the sterile syringes were stored at 4°C (in the dark) until administration. Care was taken to ensure that the antibody remained in solution prior to administration.

**Animal challenge and therapy.** SEB toxin was obtained from BEI Resources (Manassas, VA) and was accompanied by results of purity testing shown by electrophoresis. The toxin was reconstituted in phosphate-buffered saline immediately before being placed into the aerosol-generating nebulizer. Animals were exposed to the toxin using a dynamic head-only inhalation exposure apparatus controlled by an electronic flow process platform (Biaera Technologies, Hagerstown, MD) and which has been described previously (38, 39). Briefly, anesthetized animals are transported into a class III biological safety cabinet outfitted with the head-only inhalation configuration. The aerosol concentration and the estimated inhaled dose were calculated as described previously (40) using the minute volume measured during plethysmography and the SEB concentration in the aerosol as determined by protein assays of the all-glass impinger samples.

**Antibodies.** The antibodies were provided by IBT (IgG121) and Mapp (c19F1) as a liquid product in a sealed glass vacuum vial. The doses for the evaluation were derived from the starting concentration and administration of the antibody was based upon prevailing body weights of the animals enrolled in the study. During the time between the extraction and use of the antibody, the sterile syringes were stored at 4°C (in the dark) until administration. Care was taken to ensure that the antibody remained in solution prior to administration.

**Hematology, serum biochemistry, and blood coagulation.** Hematologic analysis was performed with blood samples collected in EDTA anticoagulant using a Sysmex XT-2000i analyzer, and analysis of serum from clotted samples of blood was done on an Olympus AU400 chemistry analyzer, with results automatically downloaded to the Animal Record System.

**Cytokine analysis.** Cytokine levels in serum were assayed using the Milliplex MAP primate/rhesus cytokine/chemokine polystyrene bead panel (PRCYTOMAG-40K; Millipore Corp. [Billerica, MA]). This assay provides analysis for selected cytokines and chemokines within a single sample, which are granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), transforming growth factor alpha (TGF-α), sCD40L, MIP-1β, MCP-1, IL-18, IL-17, IL-15, IL-13, IL-12/23 (p40), IL-10, IL-8, IL-6, IL-5, IL-4, IL-2, IL-1β, gamma interferon (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Briefly, collected blood serum samples from the primates are disturbed by vortex and then individually clarified through filter spin columns (catalog no. UFC30DV00; Millipore Corp.) by spinning at 12,000 × g for 4 min at room temperature. Each standard, control, or undiluted sample, in 25 μl, is added in duplicate to antibody-conjugated beads and incubated in a 96-well filter plate overnight at 2 to 8°C with shaking at 650 rpm. After 16 to 18 h, wells are washed, and 25 μl of detection antibody is added to each well. After 1 h of incubation at room temperature with shaking, 25 μl of streptavidin-phycocyanin is added to each well and incubated for 30 min with shaking. After final washes are completed, 150 μl of sheath fluid is added to each well. The plate is analyzed using a Bio-Plex 200 suspension array system (Bio-Rad). The instrument settings are as follows: 50 events/bead, 100-μl sample size, and gate settings at 8,000 to 15,000. The software used to perform the assay and analyze data is Bio-Plex Manager version 6.0, which calculates concentrations in picograms per milliliter based on the respective standard curve for each cytokine.

**Histopathology.** Tissue samples were fixed for 48 h in zinc-modified formalin (Z-Fix; Anatech, Ltd., Battle Creek, MI), dehydrated in a series of alcohols, embedded in paraffin, and sectioned at a thickness of 5 μm before deparaffinized sections were stained with hematoxylin and eosin. Sections were examined by light microscopy on a Leica DMLB microscope equipped with a Leica EC3 camera.

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

**REFERENCES**

1. Ulrich RG. 2007. Staphylococcal enterotoxin B and related toxins. Borden Institute, Washington, DC.
2. Stiles BG, Bavari S, Krakauer T, Ulrich RG. 1993. Toxicity of staphylococcal enterotoxins potentiated by lipopolysaccharide: major histocompatibil-
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W, Frolov I, Didier PJ, Weaver SC. 2013. A chimeric Sindbis-based vaccine protects cynomolgus macaques against a lethal aerosol challenge of eastern equine encephalitis virus. Vaccine 31:1464–1470. https://doi.org/10.1016/j.vaccine.2013.01.014.

39. Pincus SH, Bhaskaran M, Brey RN, III, Didier PJ, Doyle-Meyers LA, Roy CJ. 2015. Clinical and pathological findings associated with aerosol exposure of macaques to ricin toxin. Toxins (Basel) 7:2121–2133. https://doi.org/10.3390/toxins7062121.

40. Roy CJ, Pitt L. 2005. Infectious disease aerobiology: aerosol challenge methods, p 61–76. In Swearengen JR (ed), Biodefense: research methodology and animal models. CRC Press Taylor & Francis Group, Boca Raton, FL.