THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 13, pp. 9940–9951, March 23, 2012

Constitutive Production of Catalytic Antibodies to a *Staphylococcus aureus* Virulence Factor and Effect of Infection*[S]

Received for publication, December 2, 2011, and in revised form, January 24, 2012 Published, JBC Papers in Press, February 2, 2012, DOI 10.1074/jbc.M111.330043

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Background: We examined the role of catalytic antibodies in immune defense against *Staphylococcus aureus*.
Results: IgG from non-infected humans and mice hydrolyzed the *S. aureus* extracellular fibrinogen-binding protein (Efb), and the activity was reduced following infection.
Conclusion: Constitutive but not adaptive production of catalytic antibodies suggests that Efb expresses B-lymphocyte superantigenic character.
Significance: Efb superantigenic character may be a factor in *S. aureus* pathogenesis.

Antibodies that recognize microbial B lymphocyte superantigenic epitopes are produced constitutively with no requirement for adaptive immune maturation. We report cleavage of the *Staphylococcus aureus* virulence factor extracellular fibrinogen-binding protein (Efb) by catalytic antibodies produced with no exposure to the bacterium and reduction of the catalytic antibody activity following infection. IgG catalytic antibodies that specifically hydrolyzed Efb via a nucleophilic catalytic mechanism were found in the blood of healthy humans and aseptic mice free of *S. aureus* infection. IgG hydrolyzed peptide bonds on the C-terminal side of basic amino acids, including a bond located within the C3b-binding domain of Efb. Efb digested with the IgG lost its ability to bind C3b and inhibit complement-dependent antibody-mediated red blood cell lysis. In addition to catalysis, the IgG expressed saturable Efb binding activity. IgG from *S. aureus*-infected mice displayed reduced Efb cleaving activity and increased Efb binding activity compared with uninfected controls, suggesting differing effects of the infection on the antibody subsets responsible for the two activities. IgG from children hospitalized for *S. aureus* infection also displayed reduced Efb cleavage compared with healthy children. These data suggest a potential defense function for constitutively produced catalytic antibodies to a putative superantigenic site of Efb, but an adaptive catalytic response appears to be proscribed.

Specific antibodies that bind microbial antigens noncovalently are central to immune defense against microbial infections. Many examples of IgG and IgM class antibodies that catalyze the breakdown of peptide bonds in autoantigens have been reported (1–4). A single catalytic antibody molecule is reused to cleave multiple antigen molecules, and the cleavage reaction often results in permanent inactivation of the antigen (5–7). If combined with the traditional ability to recognize antigenic epitopes with sufficient noncovalent binding affinity, the proteolytic function acquires specificity for individual antigens. These features impart to catalytic antibodies antigen neutralizing potencies superior to reversibly binding antibodies. Whether catalysis is deployed by the immune system for defense against microbes, however, remains a subject of debate.

Antibodies generally develop their noncovalent antigen binding activity over days to weeks by adaptive maturational processes driven by antigen binding to B cell receptors (BCRs)[4] (antibodies expressed on the cell surface in association with signal transducing proteins). The promiscuous catalytic function of antibodies directed to small peptide "microantigens," in contrast, is expressed innately by constitutive antibodies produced with no requirement for antigen-driven maturation (8–10). An adaptive generation of catalytic antibodies as B cells differentiate may occur if the catalytic event is itself immuno-

* This work was supported, in whole or in part, by National Institutes of Health Grant AI072439, Covalent Bioscience Inc., and the Kleberg Foundation. Eric L. Brown, Yasuhiro Nishiyama, Stephanie Planque, Keri C. Smith, and Sudhir Paul have a financial interest in patents concerning catalytic antibodies to *Staphyloccus aureus* antigens and use thereof as therapeutics. Sudhir Paul has a financial interest in Covalent Bioscience Inc.
[S] This article contains supplemental Figs. S1–S3 and Table S1.
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) JN104608.
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[4] The abbreviations used are: BCR, B cell receptor; B-SAg, B cell superantigen; Bt, biotin; CDR, complementarity determining region; Efb, extracellular fibrinogen-binding protein; FR, framework region; OVA, ovalbumin; RBC, red blood cell; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

logically selectable, *e.g.* if the peptide bond cleavage reaction releases energy that is transduced productively to stimulate B cell division and clonal selection. On the other hand, the available examples of specific catalysis by intact antibody combining sites are limited to autoantigenic targets. It is conceivable, therefore, that improvement of the constitutive catalytic function is an immunologically disfavored outcome that is feasible only under conditions of dysregulated B cell physiology in autoimmune disease (11).

A small number of microbial proteins express B cell superantigen (B-SAg) determinants on their surface (12). Like promiscuous catalysis, noncovalent B-SAg binding is a constitutive antibody function that does not require B-SAg-driven adaptive B cell maturation (12). Studies on the B-SAg determinant of the HIV coat protein gp120 indicated that a subset of the constitutively produced antibodies combine the noncovalent B-SAg binding function with catalytic activity. IgMs from noninfected humans and immunologically naive mice, the first antibody class produced in the B cell maturational pathway, cleaved gp120 (13). The gp120 cleaving activity of mucosal secretory IgA was most sufficiently rapid to neutralize HIV in tissue culture (14). The reaction model entails the initial specificity-conferring noncovalent binding, nucleophilic attack on spatially accessible peptide bond carbonyl groups followed by water attack on the covalent reaction intermediate, culminating in product release and catalytic antibody regeneration. A role for constitutive catalytic antibodies in defense against microbes, therefore, is worthy of further consideration. Unlike traditional antigens, noncovalent B-SAg binding to the BCR does not generally induce differentiation of B cells into plasma cells that secrete class switched B-SAg specific antibodies (12). However, antigen-driven B cell maturation is a stochastic process. Its success depends on certain high probability events that select for mutated antibody V domains with improved antigen recognition. Some patients with prolonged HIV infection (over two decades) produced potent neutralizing antibodies directed to the gp120 B-SAg site (15). This suggests bypass immune mechanisms that might permit slow amplification of the constitutive B-SAg recognition function.

Staphylococcus aureus can reside for years on the skin and nasal mucosa in humans without causing disease (16, 17). When these barriers are breached, systemic infection with diverse manifestations can occur, including pneumonia, meningitis, osteomyelitis, endocarditis, and toxic shock syndrome. Hospital and community acquired *S. aureus* infections are a growing public health problem due to the emergence of antibiotic-resistant bacterial strains (18). *S. aureus* produces several virulence factors that contribute to its intractability and broad disease manifestations. Here we report constitutively-produced antibodies that catalyze the cleavage of extracellular fibrinogen-binding protein (Efb), a secreted *S. aureus* virulence factor that interferes with platelet aggregation, wound healing, and complement activation (19–22). The catalytic antibody activity was reduced in *S. aureus*-infected mice and children. We suggest that Efb contains a B-SAg determinant to which specific catalytic antibodies are produced as a constitutive immune function but exposure to the determinant down-regulates the B cell subset producing the catalytic antibodies.

EXPERIMENTAL PROCEDURES

Human Subjects—The studies were conducted with written informed consent and Institutional Review Board approval. Healthy human test subjects were asymptomatic for systemic *S. aureus* infection. Individual IgG preparations from the sera of 12 adult healthy humans or pooled IgG from their sera were tested (>18 years age; 6 females and 6 males). Blood from *S. aureus*-infected children presenting with community-acquired osteomyelitis was obtained at the time of admission to The Texas Children's Hospital ($n = 16$; age range 3–12 years). Bacterial isolates cultured from the patients were confirmed to be *S. aureus* and tested for antibiotic susceptibility (23). Information collected for diagnosis included medical history, duration of symptoms prior to hospitalization, prior antibiotic use, prior hospitalization history, and blood and urine chemistry and cell profiles. Control healthy children had no evidence of systemic *S. aureus* infection ($n = 12$; age range 4–6 years).

Mice—Aseptic mice were obtained from The Jackson Laboratories (4–6-week-old female BALB/c mice bred and maintained under "maximum barrier" conditions as defined by Jackson with periodic monitoring for a panel of viruses, bacteria, mycoplasma, parasites and protozoa, including *S. aureus*, see jaxmice.jax.org/health/barrier.html). Following transfer to the University of Texas-Houston Medical School Animal Facility, the aseptic mice were housed under equivalent barrier conditions until blood collection by cardiac puncture. Pooled serum from 10 aseptic mice was used for the studies. The remaining mouse studies were done using sera from mice maintained in a routine barrier facility. The effect of *S. aureus* infection was determined following intradermal inoculation of the mice with *S. aureus* (strain USA300; 1×10^8) in 50 μl of phosphate-buffered saline (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl, 2 mM KCl, pH 7.4) (24). Twenty-one days later mice were euthanized by exsanguination under anesthesia (isofluorane) and blood was collected by cardiac puncture. Infection was monitored by daily weight measurements. Maximal weight loss was observed on day 3 post-inoculation, corresponding to 1.53 ± 0.12 g (9% of the starting weight). Sera from non-infected BALB/c mice ($n = 4$) were from BioChemed (Winchester, VA).

Antibodies—IgG was purified from sera using immobilized Protein G as described previously (25). Gel filtration of IgG (0.3 mg) was done in 6 M guanidine hydrochloride (Sigma), pH 6.5, on a Superose-6 FPLC column (GE Healthcare). Column calibration with reference proteins and computation of nominal test sample mass was conducted as described (13). The 150-kDa fraction (corresponding to retention volume of 9.6–11.4 ml) was renatured by dialysis against 50 mM Tris·HCl, 100 mM glycine, pH 8.0, containing 0.1 mM CHAPS at 4 °C (2 liters × 5 changes) for 4 days. Total protein concentrations were determined using a microBCA kit (Pierce). Electrophoretic homogeneity of IgG preparations was assessed by reducing SDS-PAGE (4–20% gels) followed by Coomassie Blue staining and immunoblotting with peroxidase-conjugated goat anti-human or anti-mouse γ chain, κ chain, and λ chain antibodies (Sigma) (25). Methods for scFv preparation and purification have been described previously (26). Briefly, the library consisted of 1.4×10^7 scFvs with C-terminal His_6 and c-*myc* tags cloned in the

phagemid vector pHEN2 prepared from the peripheral blood lymphocytes of 3 patients with lupus. Soluble scFvs were purified from periplasmic extracts of HB2151 cells by metal affinity chromatography and purity was determined by electrophoresis and blotting with antibody to the His_6 tag (mass of scFv ~27 kDa).

Proteins—Efb and dispersin cloned from *S. aureus* strain Newman and *Escherichia coli* strain O42, respectively, were expressed in *E. coli* JM101 using the pQE30 vector (Qiagen, Valencia, CA) as described (22). Recombinant proteins contained a His_6 tag that permitted their purification to electrophoretic homogeneity under non-denaturing conditions to maintain antigenic integrity (22). Biotin tag (Bt) labeling of proteins was done using 6-biotinamidohexanoic acid *N*-hydroxysuccinamide ester (Pierce) as described (27). The Bt content of Bt-Efb preparations used in this study was 1.8–4.8 (mole of Bt/mol of protein; determined by the 4′-hydroxyazobenzene-2-carboxylic acid dye (Pierce)). Bt content of other protein substrates was (mol/mol): Bt-dispersin, 12.9; Bt-BSA, 9 (ThermoFisher Scientific); Bt-gp120, 1.6 (Immunodiagnostics, Woburn, MA); Bt-extracellular domain of epidermal growth factor receptor, 2.4 (from Dr. O'Connor-McCourt, Ref. 28); Bt-human FVIII C2 domain, 0.4 (from Dr. K. Pratt, Ref. 29); human Bt-prothrombin, 1.9 (from Dr. P. Thiagarajan, Ref. 30); human Bt-thyroglobulin, 26 (Sigma); chicken Bt-ovalbumin (OVA), 1.6 (Sigma); and human Bt-transferrin, 5.4 (Sigma).

Proteolysis Assays—Hydrolysis of Bt proteins was determined in duplicates by reducing SDS-PAGE (13, 14, 27). Briefly, the Bt proteins were incubated with IgG in 20 μl of cleavage assay buffer (50 mM Tris·HCl, 100 mM glycine, pH 8.0, containing 0.1 mM CHAPS and 67 μg/ml of gelatin). The reaction mixtures were boiled, subjected to reducing SDS-PAGE (20 or 4–20% gels), and blots were stained with streptavidin-peroxidase. Protein cleavage (%) was determined by densitometry as $100 \times (1 - [\text{S-IgG}]/[\text{S-DIL}])$, where [S-IgG] and [S-DIL] represent, respectively, the intact protein band intensities (in arbitrary volume units) observed for the IgG-containing reaction and control reaction containing diluent instead of IgG. In some Bt-Efb preparations, 17.5- and 13-kDa Efb fragments were observed in the diluent control reaction. Apparent kinetic parameters were determined by measuring hydrolysis by IgG (50 μg/ml) at increasing concentrations of Bt-Efb in duplicate (0.1, 0.3, 0.9, and 2.7 μM) and fitting the rate data to the Michaelis-Menten equation. To enable accurate initial velocity estimation, incubation times were adjusted to yield ~30–50% Bt-Efb hydrolysis (incubation times for 0.1, 0.3, 0.9, and 2.7 μM Bt-Efb, respectively: 1, 2, 2, and 4 h). The effect of protease inhibitors was studied by measuring non-biotinylated Efb hydrolysis in the presence of the following protease inhibitors: EDTA (2 mM), 1,10-phenanthroline (1 mM), iodoacetamide (100 μM), pepstatin A (1 μM), and diphenyl *N*-[6-(biotinamido)hexanoyl-]amino(4-amidinophenyl)methanephosphonate (compound **1**; 100 μM; prepared as described in Ref. 25). The residual activity (%) was determined using the following equation: 100 × (% hydrolysis in the presence of inhibitor)/(% hydrolysis in the absence of inhibitor), wherein % hydrolysis was determined by densitometry of silver-stained SDS-electrophoresis gels. To identify the cleavage sites, non-biotinylated Efb (0.6 μM, 12 μg/ml) incubated for 20 h with human IgG (50 μg/ml; pool from healthy adults) was fractionated by reducing SDS-PAGE (20% gel) and transferred onto a methanol-activated PVDF membrane. Individual Coomassie-stained product bands were subjected to N-terminal sequencing by Edman's degradation (Baylor College of Medicine, Protein Chemistry Core Laboratory) (13).

*Phosphonate **1** Binding*—Trypsin from porcine pancreas (Sigma) and pooled serum IgG from healthy adult humans (100 μg/ml) were incubated with phosphonate **1** (0.1 mM) for 18 h. Their phosphonate adducts were detected by SDS-PAGE under non-reducing conditions followed by streptavidin-peroxidase blotting (25).

Efb-binding Assay—Murine and human IgG binding to immobilized Efb (1 μg/well) was measured by ELISA. Bound IgG was detected using peroxidase-conjugated anti-mouse IgG (25) or alkaline phosphatase-conjugated anti-human IgG (Fc specific; Sigma) (23). In competition assays, IgG binding was measured in the presence of Efb, an irrelevant protein (OVA) or neither (diluent controls).

C3b-binding Analysis—Efb (0.1 μM) was incubated at 37 °C with IgG (0–1.25 μM) for 20 h, subjected to SDS-PAGE (20% Tricine gels) under reducing conditions, and transferred onto nitrocellulose membranes. The blots were probed with a digoxigenin-C3b conjugate followed by visualization using an alkaline phosphatase-conjugated anti-digoxigenin antibody (22, 31).

Complement Inactivation—The CH50 complement assay (Diamedix, Miami, FL) was used to determine complement activity (22). Efb (0.1 μM) was incubated for 20 h at 37 °C in cleavage assay buffer in the absence or presence of IgG (0–1 μM; 20 μl of reaction mixtures). The reaction mixtures were incubated further with reference serum supplied in the assay kit (source of complement) and then with antibody-coated red blood cells (RBCs). RBC lysis was quantified by measuring absorbance (A_{415}). The data are expressed as percent of observed RBC lysis using reference serum alone in the absence of Efb or IgG-Efb ($100 \times A_{415}$ of test sample)/A_{415} of reference serum alone).

Molecular Modeling—scFv XP-00-00-1H2 (scFv 1H2) was modeled using the following modeling servers: Prediction of ImmunoGlobulin Structure server (PIGS; arianna.bio.uniroma1.it/pigs) and RosettaAntibody Modeling (antibody.graylab.jhu.edu) (32, 33). Both are homology-based modeling programs relying on independent template structure databases that generate models by grafting complementarity determining region (CDR) loops onto selected framework region (FR) templates. The PIGS model was generated using these options: "best H and L chains template selection" and "transfer conserved + SCWRL 3.0 side chain modeling." The RosettaAntibody model was generated using the "full protocol," which refines the model using Monte Carlo sampling followed by energy minimization. The models obtained from the two servers were mostly superimposable (root mean square deviation values of the models for the C-α position and side chains, respectively, 1.47 and 2.45 Å). Potential nucleophilic dyads in the RosettaAntibody model of scFV 1H2 were identified as described (34, 35), defined as sites in which the hydroxyl oxygen

atoms of Ser or Thr are located within 4 Å from a possible general base (the imidazole nitrogen atoms of His, the β/γ-carboxylate oxygen atoms of Glu/Asp, the ϵ-amino nitrogen atom of Lys, or the ω-amino/imino nitrogen atoms of Arg). Potential nucleophilic triads contained an additional second general base within hydrogen bonding distance of the first general base.

RESULTS

Constitutive Murine Catalytic Antibodies to Efb—Initially, we studied whether antibodies capable of hydrolyzing Efb are produced by mice without stimulation by exogenous microbial antigens. Humans can carry *S. aureus* on skin and mucosal surfaces for prolonged durations often with no evidence of systemic disease. Unlike humans, mice maintained under laboratory research conditions are not natural *S. aureus* carriers (36, 37).

To eliminate the possibility of incidental exposure to *S. aureus* as the cause of antibody production, we tested pooled IgG from the serum of BALB/c bred under aseptic conditions at The Jackson Laboratory and maintained under aseptic conditions in our animal facility. The activity of purified IgG was measured using biotinylated Efb as substrate. The IgG preparations were electrophoretically homogenous. All IgG protein bands detectable by Coomassie Blue staining were also stainable by anti-IgG antibodies (supplemental Fig. S1*A*). Previous studies using identically prepared catalytic IgG preparations directed against other polypeptide substrates indicated that they were sufficiently pure to detect antibody catalytic activities without confounding effects of enzyme contaminants (1, 8, 38). As the Efb was labeled sparingly with the biotin tag (at 1.9–4.8 of 18 available Lys residues), the method does not allow visualization of small reaction products devoid of the tag. Large Bt-containing Efb fragments were detected readily following treatment with IgG (15-, 13-, and 12-kDa bands; Fig. 1*A*). Disappearance of the parent 19-kDa Bt-Efb served as the index of the overall reaction rate. The cleavage reaction was time-dependent. Complete Bt-Efb degradation was evident over 32 h (Fig. 1*B*).

Biotinylated extracellular domain of epidermal growth factor receptor, bovine serum albumin (BSA), and OVA were not cleaved by IgG from aseptic mice, indicating the absence of nonspecific proteolytic activity (Fig. 1*C*). The Efb-directed catalytic activity was saturable with increasing Bt-Efb concentrations. From the apparent V_{max} value estimated by fitting to the Michaelis-Menten-Henri equation (1.02 μM Bt-Efb/μM IgG/h, Table 1), degradation of 343 μM Efb/μM IgG is predicted over 2 weeks (corresponding to the approximate half-life of IgG in blood). This value exceeds by more than 170-fold the maximum Efb neutralizing capacity of reversibly binding IgG (2 μM Efb/μM IgG assuming bivalent binding). Apparent K_m of the IgG for Efb was 0.96 μM, suggesting a moderate noncovalent Efb binding strength (for catalysts, a small K_m value indicates comparatively strong noncovalent binding affinity; K_m of classical enzymes for polypeptides is generally in the high micromolar-millimolar range, see Ref. 39).

In addition to catalysis, the purified IgG displayed binding to Efb by ELISA tests at levels superior to control BSA antigen

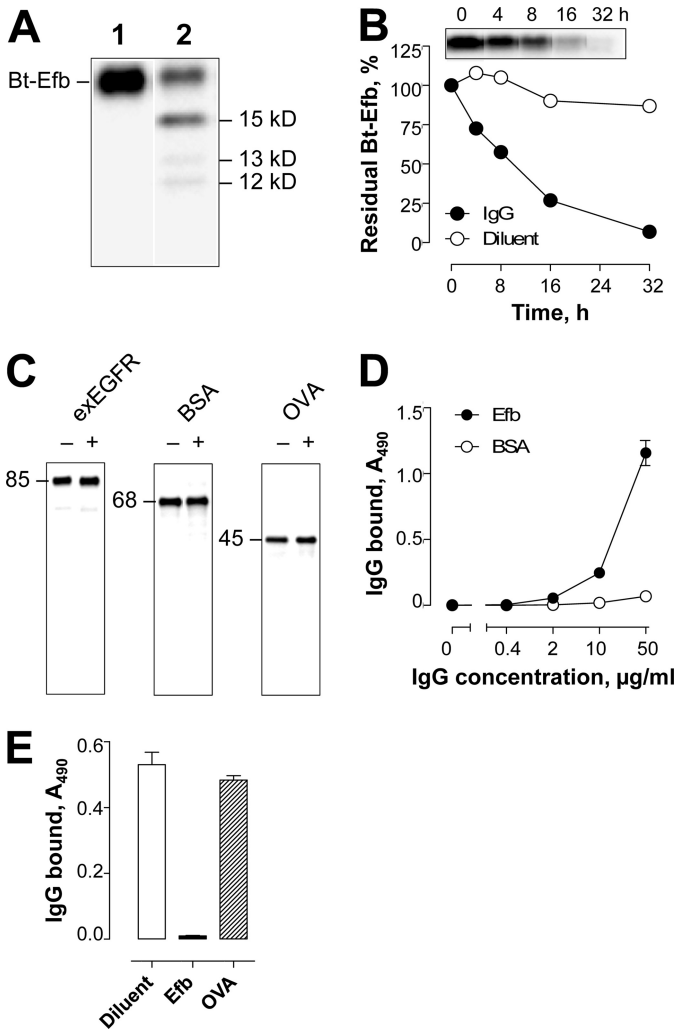


FIGURE 1. **Efb cleavage and binding by IgG from aseptic mice.** *A,* Bt-Efb cleavage. Shown are Bt-Efb (100 nM) product profiles determined by SDS-PAGE and streptavidin-peroxidase staining. Bt-Efb was incubated in the absence (*lane 1*) and presence (*lane 2*) of IgG (150 μg/ml; purified from a pool of sera from 10 mice) for 24 h at 37 °C. *B,* time-dependent Bt-Efb cleavage. Bt-Efb (2.7 μM) was treated with IgG (50 μg/ml) for varying durations. Residual intact Bt-Efb was measured by electrophoresis and densitometry. *Inset*, the intact Bt-Efb band region of the streptavidin-peroxidase stained blot (19 kDa). *C*, lack of cleavage of human epidermal growth factor receptor extracellular domain (*exEGFR*), bovine serum albumin (*BSA*), and OVA. Individual biotinylated proteins (100 nM) were treated with IgG as described in *panel A*. *Labels above lanes* denote incubations with diluent (−) or IgG (+). *D,* Efb binding. IgG binding to immobilized Efb or BSA (1 μg/well) was measured by ELISA in duplicates. *E*, inhibition of IgG-Efb binding by Efb. IgG (15 μg/ml) binding to Efb was determined as in *panel D* in the absence or presence of Efb or OVA (3 μg/ml).

(Fig. 1*D*). Binding of immobilized Efb was inhibited competitively by soluble Efb but not by a control polypeptide, confirming specificity (Fig. 1*E*). It may be concluded that the aseptic mice produce both Efb cleaving and binding antibodies.

Human Efb-directed Antibodies—Next, we measured Bt-Efb cleavage by electrophoretically homogeneous IgG preparations purified identically from the blood of 12 healthy adult humans asymptomatic for systemic *S. aureus* infection (supplemental Fig. S1*B*). All 12 IgG preparations cleaved Bt-Efb, generating identical Bt-Efb product profiles (see examples in Fig. 2*A*). The Efb product profiles generated by IgG from humans and aseptic mice were also identical (compare Fig. 1*A* with 2*A*). The mag-

TABLE 1

Kinetic parameters for Bt-Efb hydrolysis by IgG from aseptic mice

Apparent kinetic constants were obtained from the least square fit of the rate *versus* Bt-Efb concentration data to the Michaelis-Menten equation (r^2 0.95).

K_m, M	V_{max}, M min^{-1} [a]	V_{max}/K_m, min^{-1} [a]	Computed Efb hydrolysis after 14 days[a,b]	Maximum Efb binding at equilibrium[a,b]
$(0.96 \pm 0.30) \times 10^{-6}$	$(0.017 \pm 0.002) \times 10^{-6}$	1.77×10^{-2}	343×10^{-6} M	2×10^{-6} M

[a] At 1 μM IgG.

[b] For illustration, included are computed values of cumulative Efb hydrolysis over 14 days and values of the maximum Efb binding by catalytic and non-catalytic IgG antibodies with equivalent binding strength.

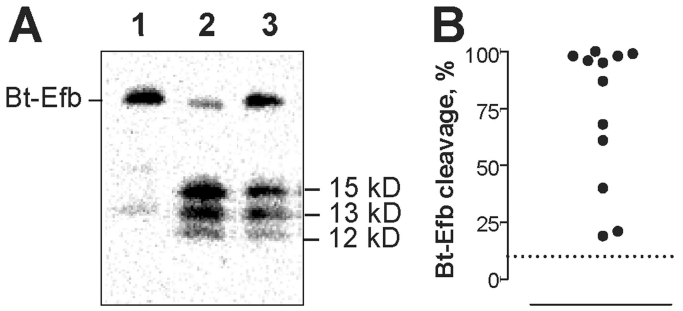


FIGURE 2. **Efb cleavage by IgG purified from sera of healthy humans.** *A*, streptavidin-peroxidase-stained blot of SDS gels showing Bt-Efb (100 nM) treated with diluent (*lane 1*) and two representative IgG preparations (53 μg/ml; purified from human subjects 2644 and 2651; *lanes 2* and *3*) for 20 h. *B*, scatter plot of Bt-Efb hydrolysis by IgG preparations from 12 healthy humans. Bt-Efb cleaving activity measured as in *panel A* was expressed as % of Bt-Efb hydrolysis. Each *symbol* represents an IgG preparation. *Horizontal dotted line* denotes the detection limit of the assay.

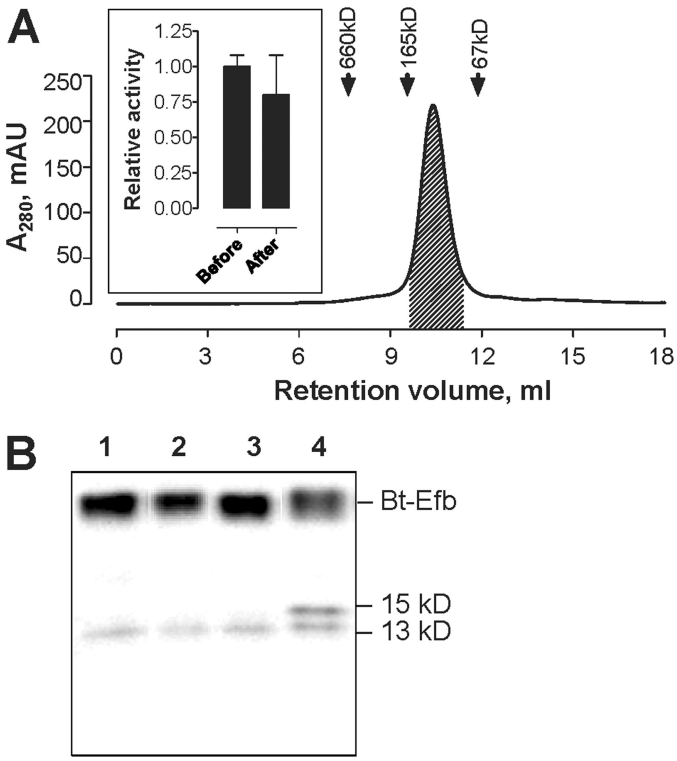


FIGURE 3. **Attribution of catalytic activity to antibodies.** *A*, denaturing gel filtration chromatogram of the Protein G-purified adult human IgG. IgG (0.3 mg) was chromatographed in 6 M guanidine hydrochloride, pH 6.5, on a Superose-6 column (A_{280} detection). *Arrows* show retention volumes of reference proteins (thyroglobulin, 660 kDa; human myeloma IgG3, 165 kDa; albumin, 67 kDa). *Inset*, relative Efb-hydrolyzing activity of the renatured 150-kDa IgG fraction from the column compared with the starting Protein G-purified IgG. Bt-Efb hydrolysis was measured as described in the legend to Fig. 1*A*. Values are mean $\pm$ S.D. of duplicates expressed relative to the catalytic activity of the starting IgG (cleavage observed at 50 μg/ml IgG, 30 $\pm$ 6%). *B*, Efb-hydrolyzing scFv clone. Streptavidin-stained blot of SDS gels showing Bt-Efb incubated with diluent (*lane 1*), a negative scFv clone (1C9, *lane 3*), a positive scFv clone (1H2, *lane 4*), or an identically purified extract of bacteria transformed with the empty vector devoid of the scFv insert (*lane 2*). Depletion of the parent Bt-Efb band and appearance of the 15-kDa fragment band is visible in *lane 4*. scFv clones were purified by binding of the His_6 tag to metal affinity columns. Reaction conditions: Bt-Efb, 100 nM; 20 h incubation.

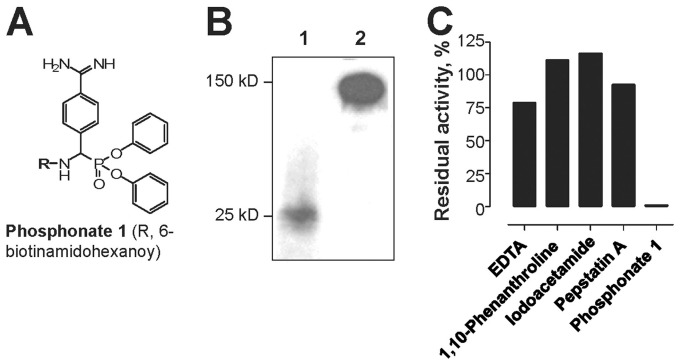
nitude of the IgG catalytic activity from the 12 humans ranged from cleavage of all available Efb to low-level Efb cleavage (Fig. 2*B*), suggesting varying catalytic antibody production.

Unlike the comparatively promiscuous peptide bond cleaving activity of classical proteases, the property of polypeptide-specific proteolysis is typical of antibodies capable of specific noncovalent antigen recognition (6, 13, 14, 40). As observed for IgG from aseptic mice, human IgG did not cleave irrelevant polypeptides under conditions permitting readily detectable Efb cleavage, including autoantigenic polypeptides that serve as substrates for catalytic antibodies from patients with autoimmune disease (supplemental Fig. S2) (38, 41).

To confirm that the specific Efb cleaving activity can be attributed to the IgG, we applied the following additional test. The IgG was subjected to resolutive gel filtration in a denaturing solvent, a procedure shown in previous studies to remove any antibody-associated adventitious protease contaminants (8, 13, 14). Consistent with its electrophoretic purity (supplemental Fig. S1*B*), the Efb-cleaving IgG eluted as a single 150-kDa peak from the denaturing gel filtration column (Fig. 3*A*). Following renaturation, the eluted IgG displayed Bt-Efb cleaving activity that was nearly identical in magnitude to the IgG cleaving activity loaded on the column (Fig. 3*A*, *inset*). Thus, the IgG catalytic activity met the classical test of purification to constant catalytic activity.

To assess whether Efb cleavage was a function of antibody V domains, we screened for Efb cleavage by 39 randomly-picked human, single chain Fv clones purified to electrophoretic homogeneity from bacterial extracts by metal-affinity chromatography (scFv, antibody V_L and V_H domains linked by a flexible peptide). The clones were obtained from a library prepared from patients with systemic lupus erythematosus. The same library and purification methods were employed previously to isolate and purify scFv clones with well defined reversible binding and catalytic activities directed to other polypeptides (26, 42, 43). Screening of 39 randomly picked scFv clones identified the Bt-Efb hydrolyzing scFv clone 1H2 (Fig. 3*B*). The identically purified extract of bacteria transformed with the empty vector without the scFv insert did not cleave Bt-Efb (Fig. 3*B*).

Thus, purified full-length IgG antibodies and scFv clones displayed Efb cleaving activity, identically purified antibody preparations of the antibodies expressed variable activity levels, the antibody activity was not diminished upon denaturing chro-



Phosphonate 1 (R, 6-biotinamidohexanoy)

FIGURE 4. **Binding and inhibitory activity of phosphonate 1.** *A*, structure. Phosphonate **1** is the biotinylated analog of diphenyl (benzyloxycarbonyl-)amino(4-amidinophenyl)methane phosphonate that binds covalently to serine protease nucleophilic sites and inhibits enzyme activity. *B*, IgG-inhibitor adducts. Shown are streptavidin-stained blots of SDS gels containing reaction mixtures of phosphonate **1** (0.1 mM) treated for 18 h with trypsin (*lane 1*, 100 μg/ml) or IgG (*lane 2*, 100 μg/ml). *C*, inhibition of Efb cleavage by protease inhibitors. Efb was incubated with adult human IgG (150 μg/ml) in the presence or absence of inhibitors of metalloproteases (EDTA, 2 mM; 1,10-phenanthroline, 1 mM), cysteine proteases (iodoacetamide, 100 μM), aspartic/glutamic acid proteases (pepstatin A, 1 μM), and serine proteases (phosphonate **1**, 100 μM). Plotted are residual IgG catalytic activities observed after inhibitor treatment. Efb hydrolysis observed in the absence of inhibitors was 78% (reaction conditions: 20 h incubation, 700 nM Efb).

TABLE 2
scFv 1H2 V_H and V_L sequence properties

Family assignments were according to the Kabat system. Germ line gene assignment was with IgBLAST. R/S ratios exclude mutations attributable to V(D)J junctional diversification. Analysis of the V(D)J gene junctions was according to IMGT/V-QUEST. The GenBank number for scFv 1H2 is JN104608.

Property	scFv 1H2	
	VL	VH
V gene family	VK4	VH4
Germline genes	V: IGKV4–1 J: IGKJ2*01	V: IGHV4–61*02 D: IGHD4–4*01 J: IGHJ5*02
V gene R/S mutation ratios		
CDRs	0/0	1/1
FRs	2/0	1/1
V(D)J junctional deletions/insertions, nucleotides	1/0	17/6

matographic fractionations, and the activity was selective for Efb. Taken together, the observations confirm the Efb cleaving activity as a *bona fide* antibody V domain property.

Proteolytic Mechanism—Previous structural studies using site-directed mutagenesis, crystallography, and selective protease inhibitors have indicated that proteolytic antibodies predominantly utilize serine protease-like nucleophilic sites to hydrolyze peptide bonds (26, 44, 45). We used the electrophilic phosphonate diester **1** (Fig. 4*A*) to probe for nucleophilic antibody species present in a human IgG preparation. This compound inhibits the active site of serine proteases by forming covalent complexes with the enzymes, *e.g.* trypsin in Fig. 4*B*, *lane 1* (25, 46, 47). Irreversible phosphonate **1**-IgG complexes were evident by denaturing electrophoresis of boiled reaction mixtures (Fig. 4*B*, *lane 2*). Phosphonate **1** inhibited the IgG catalyzed Bt-Efb cleavage reaction completely (Fig. 4*C*). Inhibitors of other classes of proteases did not inhibit the reaction appreciably (*i.e.* metalloprotease inhibitors EDTA, 2 mM, and 1,10-phenoanthroline, 1 mM; cysteine protease inhibitor iodoacetamide, 0.01 mM; and aspartic/glutamic-protease inhibitor pepstatin, 0.001 mM). The concentrations of inhibitors tested here are sufficient to completely inactivate the corresponding proteases (48). The data suggested that a serine protease-like nucleophilic mechanism is responsible for the observed IgG-catalyzed cleavage of Efb.

Identifying the precise structural features underlying the noncovalent antibody-Efb binding and subsequent peptide bond cleavage steps will require future studies. However, molecular modeling of the catalytic scFv clone 1H2 has provided initial supporting evidence for the nucleophilic catalytic mechanism (GenBank™ accession number JN104608). The scFv model contained 1 triad and 2 dyads composed of a side chain hydroxyl group with potentially enhanced nucleophilic reactivity due to hydrogen bonding to a spatially neighboring general base (supplemental Fig. S3 and Table S1; *e.g.* a side chain oxygen of Asp/Glu can serve as the general base). Similar triads and dyads containing nucleophilic Ser/Thr residues bonded intramolecularly to a neighboring general base of His, Lys, Arg, Glu, or Asp serve as the catalytic sites of serine acylases (34, 49–51). The detection of nucleophilic sites in the scFv model with the potential of participating in the catalytic reaction is consistent with the nucleophilic catalytic mechanism evident from the electrophilic inhibitor studies.

As to noncovalent Efb recognition, constitutive production of antibodies to B-SAg determinants is free of the requirement for B-SAg-driven adaptive sequence diversification of the V regions, and the recognition is thought to be dominated by amino acids located in the FRs (52, 53). Sequence comparisons with the source germline V genes indicated that the V_L/V_H region segments of the Efb cleaving scFv 1H2 contained minimal replacement mutations (aggregate of 4 replacements; Table 2). Antibodies subjected to antigen-driven adaptive diversification generally contain a higher number of V_L/V_H region replacements, *e.g.* an average of 15.1 replacements per antibody for the panel of antibodies to microbial antigens in Ref. 54. Similarly, the average number of replacements per scFv clone in the library was 13.3 ($n = 20$ clones sequenced). Three of the 4 replacements in the Efb-cleaving scFv are located in the FRs, and the R/S mutation ratio for the FRs was greater than for the CDRs (respectively, 3/1 and 1/1). In comparison, antibodies with traditional antigen binding sites contain replacement mutations located preferentially in the CDRs, and the R/S ratio for the CDRs is greater than observed for the FRs (55).

Cleavage Sites and Efb Inactivation—As the Bt-Efb substrate is labeled sparingly with biotin, some of its fragments may not contain biotin. Such fragments will not be detected by the streptavidin staining method used in the foregoing studies. Indeed, Coomassie Blue-stained electrophoresis gels containing Efb digested with human IgG revealed 4 small Efb fragments (11, 8, 6, and 5 kDa) in addition to the 15-, 13-, and 12-kDa fragments detected by streptavidin staining (Fig. 5*A*). The 7 electrophoresis bands were subjected to 10 cycles of N-terminal amino acid sequencing. Each band contained at least one N-terminal peptide sequence derived from the Efb sequence. No non-Efb peptide sequences were detected. Six N-terminal peptide sequences corresponding to internal Efb regions were identified from 6 electrophoresis bands (15, 13, 12, 11, 8, and 5 kDa). These sequences belong to products generated by IgG

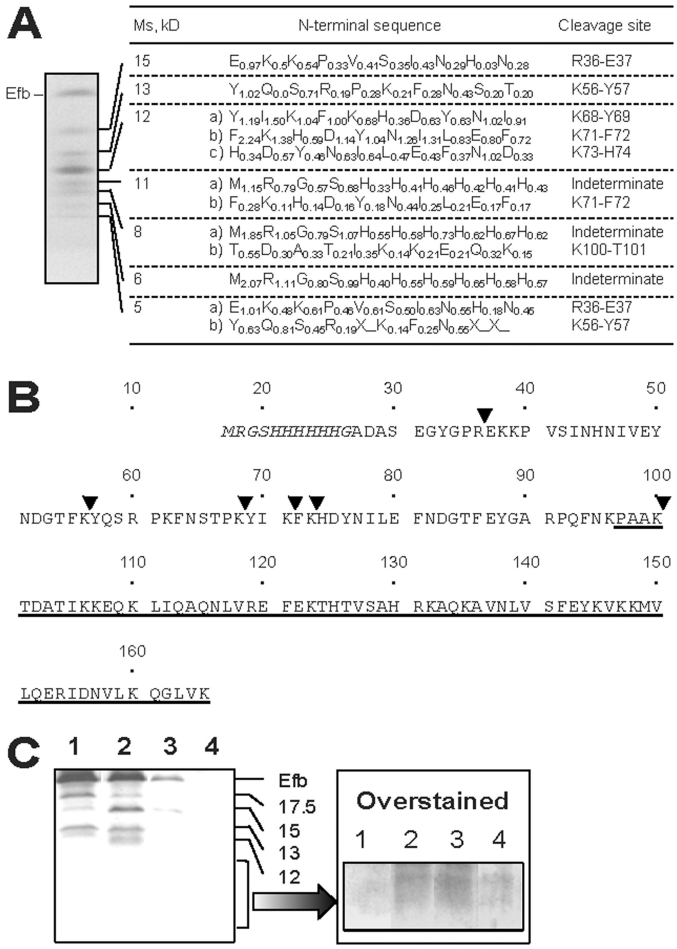


FIGURE 5. **Efb cleavage sites.** *A*, N-terminal sequences of Efb fragments generated by IgG hydrolysis. Product fragments identified in the Coomassie Blue-stained blot of a reducing SDS-gel (10%) containing the reaction mixture of Efb (12 μg/ml) incubated for 20 h with purified adult human IgG (57 μg/ml) were subjected to N-terminal amino acid sequencing (10 residues). The deduced cleavage sites are indicated. *Subscript* values indicate recovery of amino acids in picomole. *B*, sequence of Efb showing peptide bonds cleaved by human IgG. Cleavage sites are indicated by *arrows*. The *italicized* region at the N terminus represents the His_6 tag (MRGSHHHHHHG) that replaces the 26-residue signal peptide, MKNALIAKSLLTLAAIGITTTTIAST. The *underlined* region represents the C3b-binding region of Efb. For consistency with other reports in the field, the Efb amino acid numbering system conforms to the native Efb sequence containing the signal peptide. *C*, utilization of large mass Efb fragments for further cleavage reactions. Silver-stained SDS gel showing Efb (1 μM) treated for 20 h with diluent (*lane 1*) or varying human IgG concentrations for 20 h as in *panel A* (*lane 2*, 18 μg/ml; *lane 3*, 57 μg/ml; *lane 4*, 200 μg/ml). *Right box* shows an overstained version of the low mass gel area (<6 kDa). Disappearance of the large mass Efb fragments at increased IgG concentrations is evident. The 17.5- and 13-kDa bands in *lane 1* are Efb fragments present in the starting protein preparation.

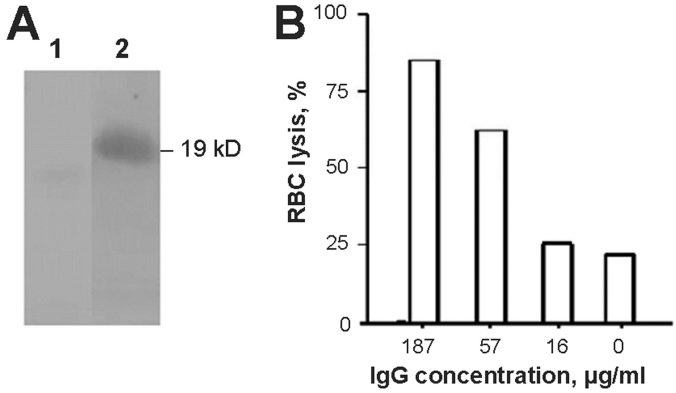


FIGURE 6. **Effect of catalytic IgG on C3b binding and Efb-mediated complement inhibition.** *A*, C3b blot of IgG-treated Efb. Efb (0.1 μM) incubated with adult human IgG (150 μg/ml, 20 h) was subjected to SDS-PAGE, transferred onto nitrocellulose paper, and allowed to bind digoxigenin-labeled C3b. *B*, attenuated Efb inhibition of complement-dependent antibody mediated RBC lysis. Efb (0.1 μM) was preincubated in the absence or presence of varying concentrations of the IgG (20 h, 37 °C). Complement activity was measured as described under "Experimental Procedures."

catalyzed cleavage at the Arg^{36}–Glu^{37}, Lys^{56}–Tyr^{57}, Lys^{68}–Tyr^{69}, Lys^{71}–Phe^{72}, Lys^{73}–His^{74}, and Lys^{100}–Thr^{101} (Fig. 5*B*). All cleavage reactions detected occurred on the C-terminal side of a basic residue (Lys/Arg). This is consistent with previous reports of preferential cleavage at peptide bonds containing basic residues at the P1 position by proteolytic antibodies to other antigens (6, 14). Three electrophoresis bands contained a product with an N-terminal sequence identical to the Efb N terminus (11-, 8-, and 6-kDa bands). As the C terminus of these products was not identified, the cleavage site is indeterminate (Fig. 5*A*). Peptides with an identical N-terminal sequence but differing mass were detected (*e.g.* the Arg^{36}–Glu^{37} sequence in the 15- and 5-kDa bands), suggesting that certain cleavage products were susceptible to further IgG-mediated cleavage. Use of conditions favoring more extensive Efb cleavage (elevated IgG concentration, prolonged incubation time) provided additional evidence that the large mass fragments are used as substrates for further cleavage. A silver-stained gel of overloaded Efb treated with 200 μg of IgG/ml revealed virtually complete disappearance of the 15-, 13-, and 12-kDa Efb fragments and appearance of a 2–6-kDa smear (Fig. 5*C*). Complete disappearance of the large mass fragments was also evident following incubation of Efb with 150 μg of IgG/ml for 72 h (not shown).

Efb binding to C3b is reported to inhibit both the classical and alternative complement activation pathways and to reduce C3b deposition on the *S. aureus* surface, thereby limiting opsonophagocytic clearance of the bacterium (22). To determine inactivation of the C3b binding function, we probed blots of Efb digested with a 150 μg of IgG/ml with digoxigenin-labeled C3b. Under comparable conditions, Efb digestion proceeded to near completion, and no Efb fragments >8 kDa were observed (Fig. 5*C*). No binding of the labeled C3b was detected, whereas C3b binding to intact Efb was readily detectable (Fig. 6*A*). Next, as a direct test for complement activation, we tested the ability of Efb digested with increasing IgG concentrations to inhibit complement-dependent lysis of RBCs mediated by anti-RBC antibodies. Progressive loss of the ability of the Efb to inhibit complement-dependent RBC lysis was evident (Fig. 6*B*), indicating that the Efb fragments generated by IgG digestion did not inhibit complement activation. The Lys^{100}–Thr^{101} bond sensitive to IgG cleavage is located within the C3b-binding Efb domain (31, 56). Efb residues 96 and 100 along with additional residues on the C-terminal side of the 100–101 scissile bond have been suggested as C3b contact sites (56, 57).

Effect of S. aureus Infection—Specific antibodies are generally produced only after B cell stimulation by foreign antigens. We determined the Efb cleaving activity of purified IgG preparations from mice infected intradermally for 21 days with *S. aureus* strain USA300 or control non-infected mice. Efb cleav-

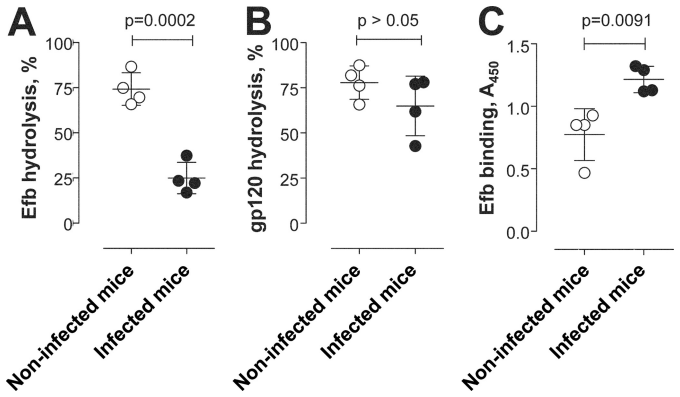


FIGURE 7. **Efb cleavage and binding by IgG purified from *S. aureus*-infected mice and non-infected controls.** *A*, Bt-Efb cleavage by IgG from sera of control mice or *S. aureus*-infected mice 21 days after bacterial inoculation. Reaction conditions were: Bt-Efb, 100 nM; IgG, 100 μg/ml; for 18 h. *B*, Bt-gp120 cleavage by the IgG samples described in *panel A*. Assay conditions are as described in *panel A*. *C*, Efb binding by immobilized Efb by IgG from non-infected mice and IgG from infected mice (4 mice each, 100 μg/ml) was measured by ELISA. The binding was inhibited by >95% in the presence of 0.16 μM soluble Efb, indicating specific Efb recognition by the IgG. Each *symbol* represents an IgG preparation from a different mouse. *p* values were determined using the unpaired, two-tailed *t* test.

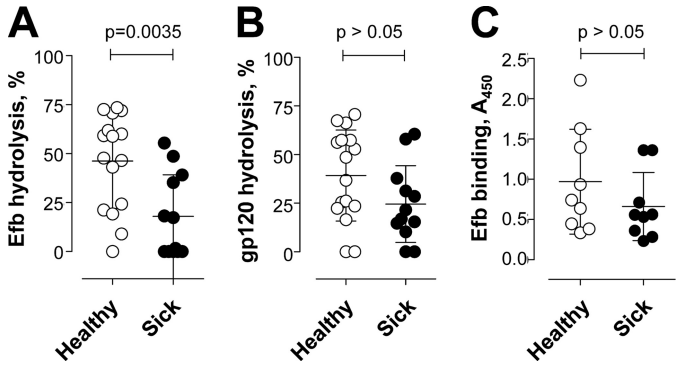


FIGURE 8. **Efb cleavage and binding by IgG purified from children hospitalized for *S. aureus* infection and control healthy children.** *A*, scatter plot of Efb hydrolysis by IgG from individual children measured by the SDS-PAGE assay. Reaction conditions were: Bt-Efb, 100 nM; IgG, 50 μg/ml; for 72 h. *B*, HIV gp120 cleavage. Bt-gp120 hydrolysis by the IgG samples described in *panel A* was measured. Assay conditions were as in *panel A* except that the incubation time was 24 h. *C*, Efb binding. Efb binding was measured by ELISA as described in the legend to Fig. 7*C*. Each *symbol* represents an IgG preparation from a different subject. *p* values were determined using the unpaired, two-tailed *t* test.

age by the IgG preparations from infected mice was reduced compared with IgG from control mice (Fig. 7*A*; $p < 0.0002$, unpaired, two-tailed *t* test). The same IgG preparations were also tested for hydrolysis of HIV protein gp120 expressing an irrelevant B-SAg determinant. Constitutively produced IgM catalytic antibodies cleave gp120 rapidly, and the catalytic activity is also detected at the concentrations of IgG tested in the present study (13). IgG from non-infected and *S. aureus*-infected mice cleaved gp120 at equivalent rates (Fig. 7*B*). The negative effect of infection on production of catalytic antibodies is restricted, therefore, to the *S. aureus* protein Efb. IgG from infected mice displayed superior binding activity to immobilized Efb compared with IgG from control, non-infected mice as determined by ELISA (Fig. 7*C*). The IgG binding activity from both mouse groups was inhibited competitively by soluble Efb (by >95% at 0.16 μM Efb). The discrepant infection-induced changes in IgG catalytic and binding activities suggests that exposure to the bacterium may induce differing functional effects on the B lymphocytes responsible for producing the subsets of catalytic and reversibly binding antibodies.

To determine the effect of *S. aureus* infection in humans, we measured Efb cleavage and binding by IgG samples purified from the sera of 16 children obtained upon hospitalization due to systemic *S. aureus* infection and 12 children with no evidence of a systemic *S. aureus* infection. IgG from children in the disease group displayed significantly reduced Efb cleavage ($p = 0.0035$ compared with the disease-free controls, unpaired, two-tailed *t* test; Fig. 8*A*). IgG from the two groups of children cleaved HIV gp120 expressing the irrelevant B-SAg determinant at statistically indistinguishable rates ($p > 0.05$; Fig. 8*B*), indicating that the infection-induced reduction of Efb cleavage activity was not a nonspecific effect. The human IgG cleavage data are consistent with the mouse intradermal infection studies. However, IgG from the systemically infected children did not display increased Efb binding compared with the disease-free group ($p > 0.05$; Fig. 8*C*). Importantly, inter-species comparisons of the effect of infection may be confounded by the differing *S. aureus* carriage status of humans and mice (see "Discussion") and differing sites of infection in the human and mouse studies.

DISCUSSION

Like noncovalent B-SAg binding, the ability to hydrolyze peptide bonds promiscuously is thought to be a constitutive property of the V domains acquired over millions of years of phylogenic antibody evolution (58). Our studies show that specific catalytic antibodies to Efb are produced constitutively without exposure to *S. aureus*. Specificity of the catalytic antibodies for Efb likely derives from the initial noncovalent binding step. Protease inhibitor and V domain modeling studies suggest that the Efb-cleaving antibodies contain activated nucleophiles responsible for the peptide bond cleavage step, akin to the serine protease family of enzymes. The same peptide bond cleaving mechanism has been described for antibodies with promiscuous proteolytic activity and autoantibodies with specific autoantigen cleaving activity (2, 6, 26, 59).

Efb is suggested to play a role in tissue colonization by *S. aureus*, retardation of wound healing in infected subjects, and inhibition of complement dependent immune defense (20, 22). The catalytic antibodies may exert a protective effect at the mucosal and systemic sites of infection. Future studies are necessary to delineate the existence of physiological inhibitors and activators of catalytic antibodies at the infection sites. In a previous study, serum did not inhibit antibodies that use the same nucleophilic catalytic mechanism as the Efb cleaving IgG (7). We have also verified the occurrence of additive Efb cleavage by purified IgG mixed with human serum (1% v/v). From our aseptic and *S. aureus*-infected mouse studies, Efb is recognized by constitutively produced catalytic antibodies, but the B lymphocytes are unable to mount an adaptive catalytic antibody response to this protein. The antibodies cleaved several peptide bonds in Efb, causing loss of complement binding and complement inhibition function of Efb. The constitutively produced Efb cleaving antibodies may furnish limited protection against

the spread of *S. aureus* infection. However, the beneficial effect of constitutive catalytic immunity may be outweighed by the impaired adaptive antibody response to Efb. Mechanistic details underlying constitutive catalytic Efb antibody production and the failed adaptive response remain to be elucidated. Nonetheless, the central findings of the present study can be explained by hypothesizing that Efb expresses a B-SAg determinant. The best studied B-SAg determinants are expressed by *S. aureus* Protein A and HIV gp120 (12). Constitutive antibodies to these proteins are well known. However, Protein A and gp120 do not stimulate the production of adaptively matured, class-switched antibodies directed to their B-SAg determinants under physiological circumstances (60–62). Noncovalent antibody binding to B-SAg determinants is dominated by FR amino acids encoded by the germline V region genes (52, 53), which may account for the failed adaptive response.

Polyclonal IgG preparations from blood are mixtures of antibodies with structurally diverse V domains. The constitutive antibody repertoire is very large, generated by pairing discrete V_L/V_H domains produced from about 150 distinct V, D, and J germline genes by V(D)J gene recombination, a step that diversifies the CDR3 sequence. Structural diversity at this stage is constitutive (or innate) in the sense that it is generated randomly without the influence of an antigen. Individual antibody subsets derived from different germline genes display differing B-SAg binding activities (63), and only a subset of antibodies with B-SAg binding activity express appreciable proteolytic activity (13). Consistent with the conclusion of their constitutive origin, the antibody subsets responsible for binding and cleaving Efb were detected by independent methods in IgG from aseptic mice. However, the acute effect of *S. aureus* infection in mice on the two antibody subsets was non-concordant. Efb cleavage was decreased, whereas binding was increased post-infection. Antigen-driven B cell maturation occurring subsequent to antigen exposure entails selection of BCRs containing CDRs that can bind the antigen, followed by further selection of the BCRs with CDR mutations that are responsible for strengthened antigen binding. In addition to the putative B-SAg determinant, Efb can be anticipated to express numerous polypeptide epitopes with the potential of stimulating an antibody response via the classical antigen-driven B lymphocyte pathway. Adaptive production of such antibodies would obscure the infection-induced effect on the constitutive binding of the B-SAg determinant. For example, HIV infection induces a vigorous antibody response to the immunodominant gp120 epitopes, even as there is no appreciable antibody response to the B-SAg determinant of this protein (60, 62). We suggest that the observed post-infection decrease of Efb cleaving activity accurately reflects the infection-induced antibody response directed solely to the putative B-SAg determinant.

The detection of catalytic antibodies to Efb in healthy humans is in accord with the conclusion of a constitutive origin of the antibodies from the aseptic mouse studies. About 40% of humans without systemic infection are persistent or intermittent carriers of *S. aureus* in the nasal mucosa (17, 19). Exposure of the immune system to polypeptide antigens via the nasal mucosa can induce mucosal and systemic antibody responses (64, 65). In contrast, mice maintained under laboratory conditions do not harbor the bacterium on their skin or mucosal surfaces for appreciable durations without evidence of disease symptoms. As discussed in the preceding paragraph, B-SAg determinants generally fail to induce an adaptive antibody response to themselves. The *S. aureus* B-SAg Protein A may even induce premature apoptosis of B cells (12, 61). As we did not establish the *S. aureus* carriage status for our human test subjects, we cannot exclude exposure to *S. aureus* as a factor responsible for variations of Efb cleavage by the IgG from healthy adult and children groups (see Figs. 2 and 8). Significantly reduced Efb cleavage but not Efb binding by IgG from children hospitalized for systemic *S. aureus* infection was evident. This indicated that the systemic spread of the bacterium is associated with a suppressive effect on catalytic antibody levels that was detected despite considerable variability of the activity between individual control children. The unusual reduction of serum Efb binding early after systemic *S. aureus* infection followed by increased Efb binding in the later stages of infection has been reported (66). Dryla *et al.* (67) observed reduced serum Efb binding activities in systemically infected humans compared with healthy controls. As noted previously, caveats in comparing the Efb binding activities of the two groups consist of a potential adaptive antibody response to the Efb epitopes devoid of B-SAg character in the systemically infected group and potential mucosal *S. aureus* exposure in the control group.

Mature antibodies generated by the classical antigen-driven selection process usually contain abundant CDR replacement mutations that enable high affinity antigen binding. Consistent with the constitutive origin of the Efb antibodies, the V regions of the Efb cleaving human scFv clone 1H2 contained only a few somatic replacements compared with the germline V gene sequences. The location of the mutations was skewed toward the FRs (3 replacements in FRs, 1 in CDRs). The scFv was isolated from an antibody library generated from the lymphocytes of patients with lupus but no evident microbial disease. Lupus is an autoimmune disease permissive for the production of antibodies with specificities that are proscribed under physiological circumstances, including antibodies specific for the HIV gp120 B-SAg determinant (68, 69). We cannot exclude the possibility that the autoimmune source of the library resulted in overrepresentation of anti-Efb scFv clones. Moreover, we did not screen the lymphocyte donors for *S. aureus* carriage status, and the possibility of antibodies generated by a chronic *S. aureus* driven immune response remains open. We reported that the physiological restriction to adaptive production of anti-B-SAg antibodies can be bypassed under certain circumstances, exemplified by antibodies to the HIV gp120 B-SAg determinant found in patients with prolonged HIV infection over two decades and in animals immunized with an electrophilic gp120 analog (15, 70). Such anti-B-SAg antibodies are distinguished by somatic replacements located preferentially in the FRs.

The Efb cleaving antibodies described in this report are of interest in understanding mechanisms that can control *S. aureus* infections. Conversely, expression of the putative B-SAg determinant by Efb may help the bacterium evade adaptive humoral immunity. *S. aureus* produces other virulence factors that facilitate bacterial adhesion to the extracellular matrix and host cell surface, exert direct toxic effects on cells, and subvert

host immunity (71–73). One such factor, Protein A, expresses a well defined B-SAg determinant that has served as the archetype for identifying novel microbial B-SAgs. Antibodies that bind additional *S. aureus* virulence factors have been reported in healthy humans (66, 67, 74). Frequent *S. aureus* exposure via mucosal surfaces and skin was assumed to account for the generation of these antibodies. Diminution of antibody binding to certain *S. aureus* virulence factors in infected humans has also been reported (66, 67). An alternative hypothesis is that some of the virulence factors express B-SAg determinants that are susceptible to constitutive catalytic antibodies but induce dysfunctional adaptive antibody responses. Insight to structural factors underlying Efb-antibody interactions is pertinent to vaccination against the bacterium. The nucleophiles responsible for catalytic activity are expressed on BCRs early in the ontogeny of B cell differentiation (75). Electrophilic immunogens that bind covalently to the BCR nucleophiles induce neutralizing antibodies to the host cell-binding site of HIV gp120 despite the B-SAg character of the site. Such an antibody response is normally "forbidden" upon noncovalent binding of B-SAg determinants to the B cells under physiological circumstances.

Acknowledgments—We thank R. Dannenbring, Dr. B. Jena, Dr. Y. Mitsuda, Dr. D. Ghosh, and R. Taniguchi for technical assistance; Dr. M. O'Connor-McCourt (Biotechnology Research Institute, National Research Council Canada) for providing the purified recombinant human extracellular epidermal growth factor receptor domain; Dr. P. Thiagarajan (Baylor College of Medicine) for providing purified human prothrombin; and Dr. K. P. Pratt (University of Washington) for providing the purified recombinant human factor VIII C2 domain.

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