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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 27, pp. 20850–20859, July 2, 2010

Development of a Two-part Strategy to Identify a Therapeutic Human Bispecific Antibody That Inhibits IgE Receptor Signaling*[S]

Received for publication, February 16, 2010, and in revised form, April 7, 2010 Published, JBC Papers in Press, May 5, 2010, DOI 10.1074/jbc.M110.113910

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The development of bispecific antibodies as therapeutic agents for human diseases has great clinical potential, but broad application has been hindered by the difficulty of identifying bispecific antibody formats that exhibit favorable pharmacokinetic properties and ease of large-scale manufacturing. Previously, the development of an antibody technology utilizing heavy chain knobs-into-holes mutations and a single common light chain enabled the small-scale generation of human full-length bispecific antibodies. Here we have extended the technology by developing a two-part bispecific antibody discovery strategy that facilitates proof-of-concept studies and clinical candidate antibody generation. Our scheme consists of the efficient small-scale generation of bispecific antibodies lacking a common light chain and the hinge disulfides for proof-of-concept studies coupled with the identification of a common light chain bispecific antibody for large-scale production with high purity and yield. We have applied this technology to generate a bispecific antibody suitable for development as a human therapeutic. This antibody directly inhibits the activation of the high affinity IgE receptor FcεRI on mast cells and basophils by cross-linking FcεRI with the inhibitory receptor FcγRIIb, an approach that has strong therapeutic potential for asthma and other allergic diseases. Our approach for producing human bispecific full-length antibodies enables the clinical application of bispecific antibodies to a validated therapeutic pathway in asthma.

Bispecific antibodies that simultaneously recognize two different antigens have significant potential clinical utility as agents that neutralize different pathogenic mediators, redirect cellular activity by recruiting different cell types to one another, or modify cell signaling by cross-linking two different receptors on the surface of the same cell (1, 2). Several groups have developed bispecific agent formats (3–5), and one of these has successfully entered human clinical trials (1, 6). However, the general application of bispecific antibodies as human therapeutics, especially for the chronic treatment of human diseases, has been hindered by the difficulties of developing antibody platforms that exhibit high stability, favorable *in vivo* pharmacokinetic properties, a lack of immunogenicity, and feasibility for large scale manufacturing and purification (5, 7–10).

Neutralization of serum IgE, which leads to the subsequent desensitization of mast cells and basophils to allergen-induced activation via down-regulation of total surface FcεRI and FcεRI signaling (11, 12), is an efficacious therapy for the treatment of moderate and severe asthmatics, including those who do not respond to any other therapies (13–15). Inhibition of FcεRI signaling by anti-IgE therapy is indirect and has a slow onset of action (11, 15) such that agents that directly and immediately inhibit FcεRI signaling have strong therapeutic potential and may be attractive alternatives to anti-IgE therapy for asthma and other allergic diseases.

Cross-linking of an activating receptor with an immunoreceptor tyrosine-based inhibitory motif-containing inhibitory receptor delivers a dominant negative signal that suppresses all signaling events downstream of the activating receptor (16–18). This approach has been applied to the high affinity IgE receptor FcεRI, and several groups have demonstrated that cross-linking FcεRI with the inhibitory receptor FcγRIIb can inhibit FcεRI activation and its downstream biology in mast cells and basophils (19–26). However, the development of a human therapeutic that cross-links FcεRI with FcγRIIb and that is suitable for chronic administration in asthma has so far been unsuccessful due to multiple factors including immunogenicity, a short *in vivo* half-life, a lack of specificity for FcγRIIb over other activating Fcγ receptor isoforms, competition by serum IgE for binding to FcεRI, and challenges for large-scale manufacturing (27).

Previously, an antibody technology was developed that enabled the efficient generation of fully human bispecific antibodies on a small scale (28). This technology consisted of sterically complementary "knobs-into-holes" mutations in the antibody heavy chain C_H3 domain that promoted heavy chain heterodimerization combined with a single common light chain that prevented heavy chain/light chain mispairing. However, large-scale production of these knobs-into-holes bispe-

* This work was fully supported by Genentech, Inc.
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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. 1–6.
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cific antibodies in mammalian cells was hindered by variable heterodimer purity.

Here we have improved and extended the knobs-into-holes, common light chain bispecific antibody format by developing a two-part antibody discovery strategy that facilitates proof-of-concept studies and clinical candidate antibody generation. The first part consists of the efficient small-scale generation of bispecific antibodies lacking a common light chain and the hinge disulfides, enabling proof-of-concept studies without the need to identify antibodies having a common light chain. The second part consists of the identification of a common light chain bispecific antibody clinical candidate for large-scale production with high purity and yield. We have applied this approach to produce a fully human bispecific antibody that cross-links FcεRI with FcγRIIb. The bispecific antibody is highly specific for FcγRIIb, is not blocked by serum IgE binding to FcεRI, and inhibits FcεRI-mediated activation of mast cells *in vitro*. Moreover, the bispecific antibody has *in vivo* pharmacokinetic properties that are comparable with normal human IgG1 antibodies produced in mammalian cells, and large-scale manufacturing of the bispecific antibody for clinical studies is feasible. Our approach for generating a human full-length bispecific antibody may be applicable to a range of clinical applications that require chronic antibody treatment.

EXPERIMENTAL PROCEDURES

Expression of 22E7/5A6 Bispecific Antibody—The 22E7-5A6 chimeric bispecific antibody was produced in *Escherichia coli* as separate heavy-light chain fragments and then annealed in a one-to-one ratio after purification using knobs-into-holes heterodimerization technology (28). The 22E7 knob (T366W) and 5A6 hole (T366S, L368A, Y407V) were expressed using separate cistrons for light and heavy chains with relative translation initiation regions of 1 and 1, as previously described (29). Each antibody fragment had cysteine-to-serine mutations in the hinge region of human IgG1 to prevent downstream purification complications.

Isolation and Annealing of 22E7/5A6 Bispecific Antibody—Each half of the bispecific antibody was independently isolated from *E. coli* using standard antibody isolation techniques. The protein was purified by protein A affinity chromatography using PROSEP-A resin (Millipore) followed by SP Sepharose Fast Flow (SP-FF) cation exchange. The protein was further purified by hydrophobic interaction chromatography using HiPropyl resin and a 0.5–0 M sodium sulfate gradient followed by chromatography on an S-200 gel filtration column. After isolation, 5 mg of 22E7 and 5 mg of 5A6 were mixed together in 10 ml of buffer containing 10 mM succinate, pH 6.0, and 0.15 M sodium chloride. The mixture was heated to 50 °C for about 10 min to anneal the two complementary heavy chains and then cooled to room temperature. The annealed product was analyzed by isoelectric focusing (IEF) using 7–10 pH range Lonza IsoGel IEF plates (Fisher). The final annealing of knob and hole results in a very strong CH3-CH3 interaction holding the two heavy chains together without the hinge disulfides (30).

Binding Specificity ELISA[2] Analysis—ELISA assays were developed to test for anti-FcεRIα, anti-FcγRIIb, and bispecific antibody specificity. To test anti-FcεRIα antibody binding specificity, human FcεRIα-coated plates were preincubated with human IgE or blocking buffer followed by incubation with anti-FcεRI antibodies. Binding of anti-FcεRIα antibodies was detected using HRP-conjugated, goat anti-human IgG Fc-specific antibody. To test anti-FcγRIIb antibody binding specificity, human FcγRIIb- or FcγRIIa-coated plates were incubated with anti-FcγRIIb antibodies. Binding of anti-FcγRIIb antibodies was detected using HRP-conjugated, goat anti-human IgG Fc-specific antibody. To test dual specificity of anti-FcεRIα/FcγRIIb bispecific antibodies, a sandwich ELISA format was used. Human FcγRIIb-coated plates were preincubated with human IgG to block the Fc-binding site of the FcγRIIb protein. Subsequently, plates were incubated with bispecific antibody followed by incubation with human FcεRIα. Dual specificity of binding was determined by detection of human FcεRIα by further incubation with biotinylated human IgE and streptavidin-HRP. All ELISA data were plotted using a four-parameter nonlinear regression curve-fitting program (Kaleidagraph, Synergy Software).

Selection of Phage Antibodies Specific for FcεRIα or FcγRIIb—Human phage antibody libraries were built on a single human framework, based on the consensus heavy chain subgroup III and the consensus light chain κI, by introducing synthetic diversity at solvent-exposed positions within the heavy chain complementary-determining regions (CDRs). The diversity of the libraries mimics natural diversity using tailored codons. Antibody Fab fragments were displayed bivalently on the surface of M13 bacteriophage particles as described previously (31). Plates were coated with human FcεRIα extracellular domain (ECD)-His-tagged protein or human glutathione *S*-transferase-FcγRIIb fusion protein (10 μg/ml) and blocked with PBST buffer (PBS, 0.05% Tween 20) supplemented with 1% bovine serum albumin. The phage libraries were added to the plates, incubated at room temperature for 2 h, and recovered after washing with PBST buffer 10 times. Recovered phage was amplified in *E. coli* XL-1 blue cells. During subsequent selection rounds, the stringency of plate washing was gradually increased (32). Unique and specific phage antibodies to human FcεRIα ECD or FcγRIIb ECD were identified using phage ELISA and DNA sequencing. To identify IgE non-blocking antibodies against human FcεRIα, plates coated with FcεRIα ECD-His proteins were preincubated with 10 nM human IgE before phage antibodies were added. Clones that could bind to FcεRIα ECD-His in the presence of IgE were selected for further characterization. Phage ELISA was also utilized to select phage antibodies that specifically bind to FcγRIIb but not FcγRIIa. Clones of interest were reformatted to full-length IgGs by cloning VL and VH regions of individual clones into LPG3

[2] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; CDR, complementary-determining region; ECD, extracellular domain; PCA, passive cutaneous anaphylaxis; SCID, severe combined immune deficiency; ERK, extracellular signal-regulated kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

and LPG4 vectors, respectively, transiently expressed in mammalian cells, and purified with protein A columns (33).

Affinity Maturation of Anti-FcεRIα Antibody—Phagemid displaying monovalent Fab on the surface of M13 bacteriophage (31) served as the library template for grafting light chain (V_L) and heavy chain (V_H) variable domains of clone 9202 for affinity maturation. Stop codons were incorporated in CDR-H2 and CDR-H3. A soft randomization strategy (50% wild type amino acid, 50% all other amino acids) was adopted for affinity maturation as described previously (32). To retain the common light chain, only selected residues in heavy chain CDRs were randomized, and two different combinations of CDR loops, H1/H2 and H1/H2/H3, were selected for randomization. As opposed to the initial phage selection strategy, which utilized bivalent Fab to increase Fab expression levels, affinity maturation was performed using monovalent Fab to minimize binding avidity effects. We affinity-matured the anti-FcεRIα antibody because initial experiments indicated that we could not achieve maximum inhibition of FcεRI signaling with the parental anti-FcεRIα Fab. Affinity maturation of the anti-FcγRIIb antibody was not necessary, as our initial anti-FcγRIIb Fab exhibited acceptable *in vitro* activity. For selecting affinity-matured clones, phage libraries were sorted against human FcεRIα-ECD, subjected to plate sorting for the first round, and followed by four rounds of solution phase sorting as described previously (31). After five rounds of panning, a high-throughput single-point competitive phage ELISA was used to rapidly screen for high affinity clones as described previously (34). Clones with a low ratio of absorbance at 450 nm in the presence *versus* absence of 10 nM human FcεRIα were chosen for further characterization. One leading affinity-optimized clone (from 90 to 14 nM) was further affinity-matured by a slight modification of the procedure described above. Specifically, three individual library templates were generated, each with stop codons introduced in CDR-H1, -H2, or -H3, respectively. Three heavy chain CDR libraries, H1, H2, and H3, were constructed for sorting, and the affinity of the leading clone was improved from 14 to 0.6 nM.

Expression of 9202.1/5411 Bispecific Antibody—The 9202.1/5411 bispecific antibody was produced in *E. coli* by co-expression of the heavy chain knob, the heavy chain hole, and the common light chain from a single expression plasmid. The expression plasmid construct consists of separate cistrons for each heavy chain and the light chain and uses relative translation initiation regions of 1 for each chain. The bispecific antibody was expressed in both 2-liter shake flasks cultures and 10-liter fermentations as previously described (29).

Isolation and Characterization of 9202.1/5411 Bispecific Antibody—Fermentation in a 10-liter reactor was performed on *E. coli* transformed with the single plasmid containing two different heavy chains and a single, common light chain. The fermentation yielded about 2.0 kg of bacterial-cell pellet that was resuspended in 5 liters of buffer containing 25 mM Tris, pH 7.5, 5 mM EDTA, and 125 mM sodium chloride. Lysis was done by three passes through a Microfluidics (Newton, MA) microfluidizer model HC80003A, and the resultant lysate was precipitated with 0.4% polyethyleneimine at 4 °C for 20 h. The precipitated lysate was centrifuged, and the supernatant was filtered before application to a protein A affinity column using MAbSuRe (GE Healthcare). The bound antibody was eluted with 40 mM citrate, pH 3.0, and the pooled eluate was further purified by cation exchange chromatography using S-FF (GE Healthcare) and a salt gradient of 0–0.5 M sodium chloride. The protein was further purified by application to cation exchange resin CM-Sepharose Fast Flow (CM-FF) (GE Healthcare) and eluted with a pH gradient from 4.5–9.2. Purified protein was analyzed by SDS-PAGE under reducing and non-reducing conditions and by electrospray ionization-time-of-flight mass spectrometry. For mass spectrometry, reduced and non-reduced antibody samples were separated by reverse phase high performance liquid chromatography using PLRP-S columns from Polymer Laboratories (Amherst, MA) followed by analysis with an Agilent 6210 time-of-flight mass spectrometry.

RBL Cell Line Generation—RBL cell line variants were generated by retroviral transduction of RBL-2H3 cells (ATCC CRL-2256) with FcεRIα, FcγRIIb1, and/or FcγRIIa (human and/or mouse). cDNA of the full-length genes was cloned into the pMSCV retroviral expression vector (BD Biosciences Clontech) either singly or in combination with an IRES (internal ribosomal entry sequence) to allow for bicistronic co-expression of two genes. Retroviruses were produced using the amphotropic PG13 packaging cell line (ATCC CRL-10686). Surface expression of transfected receptors was determined by flow cytometry, and cells expressing comparable high levels of receptors were sorted for use in experiments.

RBL Cell Activation—RBL cell line variants were incubated for 24 h at 37 °C with 5 μg/ml NP-specific human IgE (JW8.5.13, Serotec) to load FcεRI with NP-specific human IgE. Cells were activated by incubation with NP-conjugated ovalbumin for 1 h at 37 °C. Activation-associated degranulation was measured by quantitating histamine levels in the cell culture medium by ELISA (histamine ELISA kit, IBL Immunobiological Laboratories). LTC4 levels were measured by enzyme immunoassay (EIA) (leukotriene C4 EIA kit, Cayman #520211), PGD2 levels were assayed by EIA (PGD2-MOX EIA kit, Cayman #512011), and cytokines were measured using a multiplex assay kit (Rat cytokine/chemokine LINCOplex kit, Linco Research RCTYO-80K-PMX). For inhibition studies, RBL cells were preincubated with bispecific antibody for 1 h at 37 °C before activation with antigen.

RBL Biochemistry—RBL cell line variants were incubated for 24 h at 37 °C with 5 μg/ml JW8.5.13 (Serotec). Growth media was replaced with Opti-MEM (Invitrogen catalog #31985) containing 1 μg/ml JW8.5.13, and cells were cultured for an additional 3–4 h and washed 3 times with Opti-MEM before the addition of 2 μg/ml concentrations of various antibodies for 1–2 h. Cells were activated with 0.1 μg/ml NP-ovalbumin for 0, 2, 5, and 10 min. At the specified time points, the cells were lysed on ice with cell lysis buffer (radioimmune precipitation assay buffer, Sigma catalog #R0278) containing protease inhibitor mixture (Roche Applied Science catalog #11697498001) and phosphatase inhibitor mixture (Sigma catalog #P5726). Proteins in cell lysates were resolved on 8–16% acrylamide gel, transferred to nitrocellulose, and incubated with the antibodies against phospho-CD32 FcγRIIb (Cell Signaling Technologies, catalog #4141), phospho-p44/p42 map kinase (ERK) (Cell Signaling Technologies, catalog #9106), p44/p42 map kinase

(ERK) (Cell Signaling Technologies, catalog #9102), phosphotyrosine (Cell Signaling Technologies, catalog #9411), and CD32 FcγRIIb (Genentech 5A6).

Basophil Activation—Human basophils were isolated from 50 ml of blood by negative selection using Miltenyi Macs basophil isolation kits. Purity was >90% as assessed by flow cytometry. Purified basophils were activated by incubation with polyclonal goat anti-human IgE antibodies (Sigma), and activation-associated degranulation was measured by quantitating histamine levels released into the cell culture medium. For inhibition studies, basophils were preincubated with bispecific antibody for 30 min at 37 °C before activation.

Passive Cutaneous Anaphylaxis (PCA)—Human FcεRIα BAC transgenic FVB mice, 12–14 weeks old, were generated at Genentech and maintained in accordance with American Association of Laboratory Animal Care guidelines. The studies were conducted in compliance with National Institutes of Health Guide for the Care and Use of Laboratory animals and were approved by the Institutional Animal Care and Use Committee at Genentech. Mice were passively sensitized by intradermal injections of anti-NP human IgE (12.5 ng in 20 μl of PBS) in the right flank. The mice were challenged 24 h later with an intravenous injection of 200 μg of NP-ovalbumin containing 1% Evans blue (Sigma) in 200 μl of PBS (via tail vein). Fifteen minutes after the antigen challenge, the mice were euthanized, and the Evans blue dye was extracted from the flank skin (antigen-injected skin site) in 1 ml of formamide. The amount of dye in the extract was determined by measuring the absorbance of the formamide extract at 620 nm. 22E7/14H6 bispecific antibody was administered after cutaneous mast cell sensitization 2 h before mast cell activation.

Antibody Pharmacokinetic Studies in SCID Beige Mice—Pharmacokinetic properties of the 9202.1/5411 bispecific antibody and the parental 9202.1 and 5411 antibodies were assessed in SCID beige mice. Antibodies were administered to female SCID beige mice at a single intravenous dose of 10 mg/kg via a jugular cannula. Serum samples were collected at different time points over a period of 28 days and were analyzed by a colorimetric sandwich ELISA. Briefly, NUNC 384 well Maxisorp immunoplates were coated with a donkey $F(ab')_2$ anti-human IgG Fc (Jackson ImmunoResearch, West Grove, PA) antibody to capture the analytes. Standards, controls, and samples diluted in PBS containing 0.5% bovine serum albumin, 0.35 M NaCl, 0.25% CHAPS, 5 mM EDTA, 0.05% Tween 20, and 15 ppm Proclin were incubated on the plates for 2 h at room temperature. Bound analytes were detected using a horseradish peroxidase-conjugated goat $F(ab')_2$ anti-human IgG Fc (Jackson ImmunoResearch). Plates were developed using 3,3′,5,5′-tetramethyl benzidine (KPL, Inc., Gaithersburg, MD). Absorbance at 450 nm was measured on a Multiskan Ascent reader (Thermo Scientific, Hudson, NH). Concentrations were determined from the standard curve using a four-parameter nonlinear regression program. The standard curve range of the assay is 0.3–40 ng/ml in buffer. The minimum dilution for the samples is 1:10, resulting in a minimum quantifiable concentration of 3 ng/ml in serum. Values for the pharmacokinetic parameters were determined by a noncompartmental model using WinNonlin software (Pharsight Corp.).

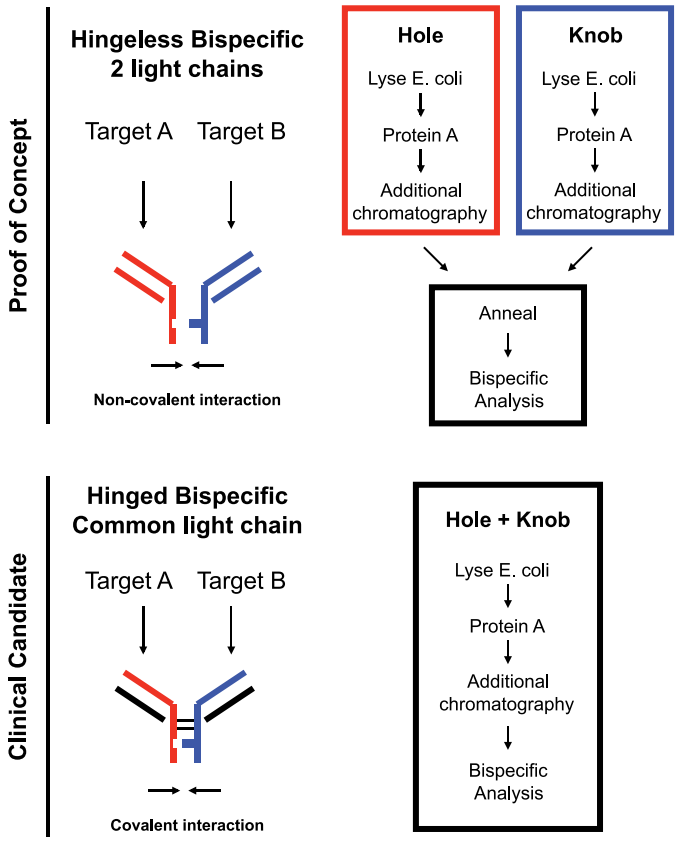


FIGURE 1. **Expression and purification schemes for the generation of proof-of-concept and human therapeutic bispecific antibodies in *E. coli*.** Proof-of-concept bispecific antibodies lacking a common light chain are produced at a small-scale as hingeless bispecific antibodies. Each arm of the bispecific antibody (knob and hole) is produced and purified separately. The two arms are then mixed together for annealing and subsequently purified and analyzed. Human therapeutic bispecific antibodies with a common light chain are produced in *E. coli* from a single plasmid containing knob and hole heavy chains and the common light chain. The intact bispecific antibody is subsequently purified and analyzed.

RESULTS

Development of a Two-staged Bispecific Antibody Discovery Strategy—We developed two different bacterial expression systems, one for the small-scale production of bispecific antibodies for proof-of-concept studies and one for the large-scale production of human therapeutic bispecific antibodies (Fig. 1). For both expression systems, knobs-into-holes heavy chain technology enabled the specific formation of antibody heterodimers. For proof-of-concept studies, we removed the need to identify a common light chain by producing the knobs and holes heavy chains and their respective light chains in separate *E. coli* cells to prevent heavy chain/light chain mispairing. In addition, each arm of the antibody heterodimer contained cysteine to serine mutations in the antibody hinge region to prevent downstream purification complications. This approach can facilitate clinical candidate identification by enabling the generation of multiple bispecific antibody variants and/or species-specific surrogate bispecific antibodies for proof-of-concept studies and to help determine optimal antibody characteristics such as affinity or epitope without the need to identify a common light chain for each bispecific antibody being tested. For the large-scale production of a human therapeutic bispe-

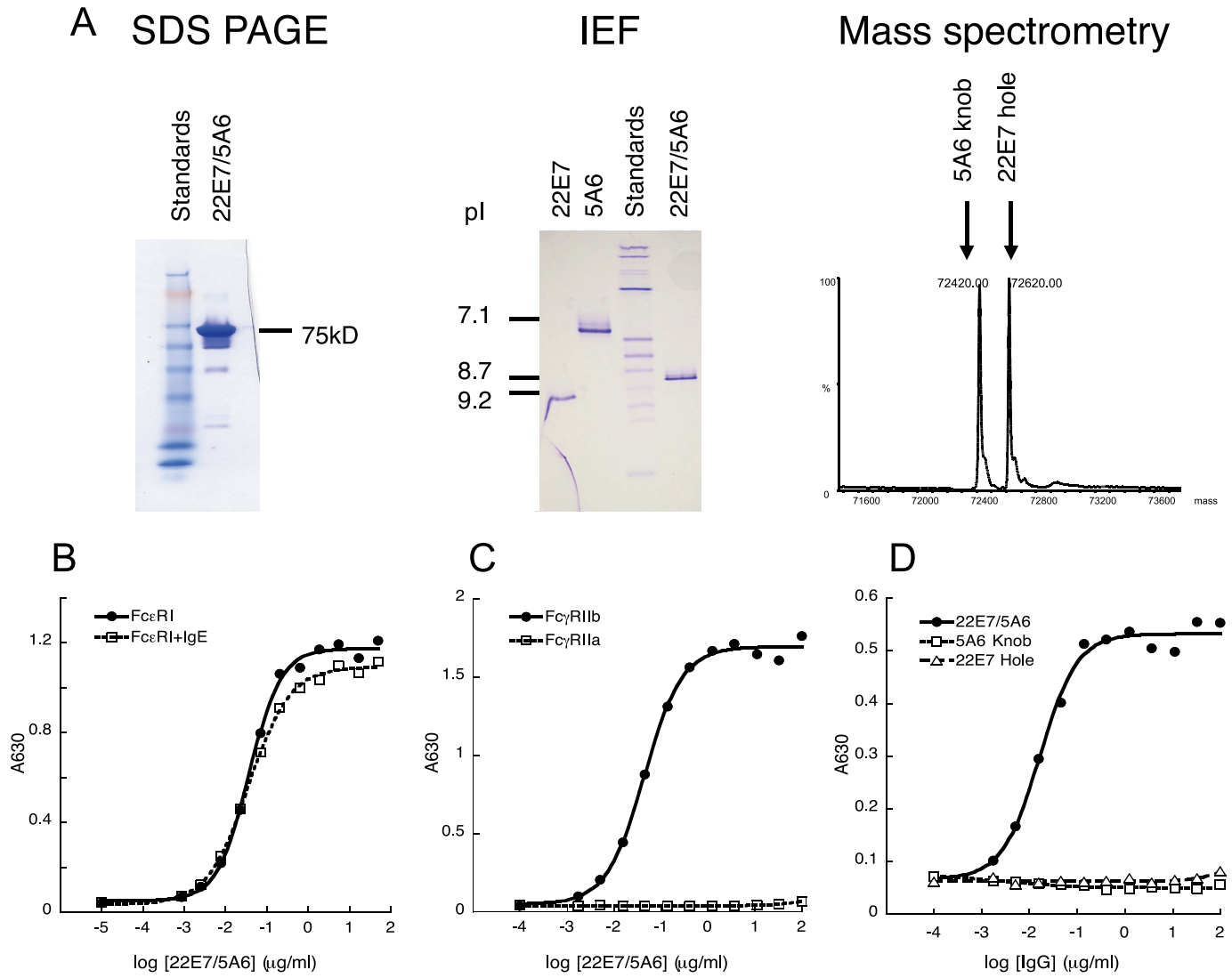


FIGURE 2. **Generation and characterization of hingeless 22E7/5A6 bispecific antibody.** *A*, shown is SDS-PAGE analysis of non-reduced 22E7/5A6 bispecific antibody shows high purity, with 22E7 hole and 5A6 knob co-migrating at an apparent molecular mass of ~75 kDa as expected. Isoelectric focusing gel (*IEF*) analysis of the 22E7/5A6 bispecific antibody preparation indicates that it consists of a single heterodimeric antibody species with no contaminating 22E7 or 5A6 homodimers. Mass spectrometry analysis of non-reduced 22E7/5A6 bispecific antibody shows high purity and the expected masses of 22E7 hole and 5A6 knob. *B*, shown is ELISA analysis of 22E7/5A6 bispecific antibody binding to recombinant human FcεRIα extracellular domain protein in the presence and absence of human IgE. *C*, shown is an ELISA analysis of 22E7/5A6 bispecific antibody binding to recombinant human FcγRIIb and FcγRIIa extracellular domains. *D*, shown is an ELISA analysis of 22E7/5A6 bispecific antibody, 5A6 knob, and 22E7 hole binding to both human FcεRIα and human FcγRIIb extracellular domains. Recombinant human FcγRIIb is directly coated on the ELISA plate followed by incubation with 22E7/5A6, 22E7, or 5A6. Bispecific binding is detected by further incubation with recombinant human FcεRIα extracellular domain followed by incubation with biotinylated human IgE binding and detection of the entire antibody/extracellular domain/IgE complex with HRP-conjugated streptavidin.

cific antibody, we generated a single plasmid containing the knob and hole heavy chains and the common light chain. This single plasmid expression scheme was adapted to a bacterial expression technology that was previously developed to enable the large-scale production of conventional monospecific antibodies in a single *E. coli* cell line (29).

We subsequently utilized these bacterial expression systems to generate bispecific antibodies that inhibit the activation of FcεRI on mast cells and basophils by cross-linking FcεRI with the inhibitory receptor FcγRIIb, an approach that has strong therapeutic potential for asthma and other allergic diseases.

A Proof-of-concept Anti-human FcεRIα/Anti-human FcγRIIb Bispecific Antibody Inhibits IgE Receptor Signaling in Mast Cells and Basophils in Vitro—To demonstrate that bispecific antibody cross-linking of FcεRI and FcγRIIb can inhibit IgE-mediated activation of mast cells and basophils, we identified two distinct monoclonal hybridoma antibodies targeting the α-subunit of FcεRI (FcεRIα) and FcγRIIb and used them to create a chimeric bispecific antibody for proof-of-concept studies. 22E7 is a previously characterized murine anti-human FcεRIα antibody that is not blocked by IgE binding to FcεRIα (35) (supplemental Fig. 1*A*). 5A6 is a murine anti-human FcγRIIb antibody that does not cross-react with FcγRIIa, the activating isoform of FcγRII that is 92% identical to FcγRIIb in the extracellular domain (supplemental Fig. 1*B*). We created a chimeric mouse/human bispecific antibody comprising the 22E7 and 5A6 Fabs and a human knobs-into-holes IgG1 Fc. Purified 22E7/5A6 bispecific antibody does not contain 22E7 or 5A6 homodimer contaminants and demonstrates bifunctional binding to FcεRIα and FcγRIIb (Fig. 2, *A–D*).

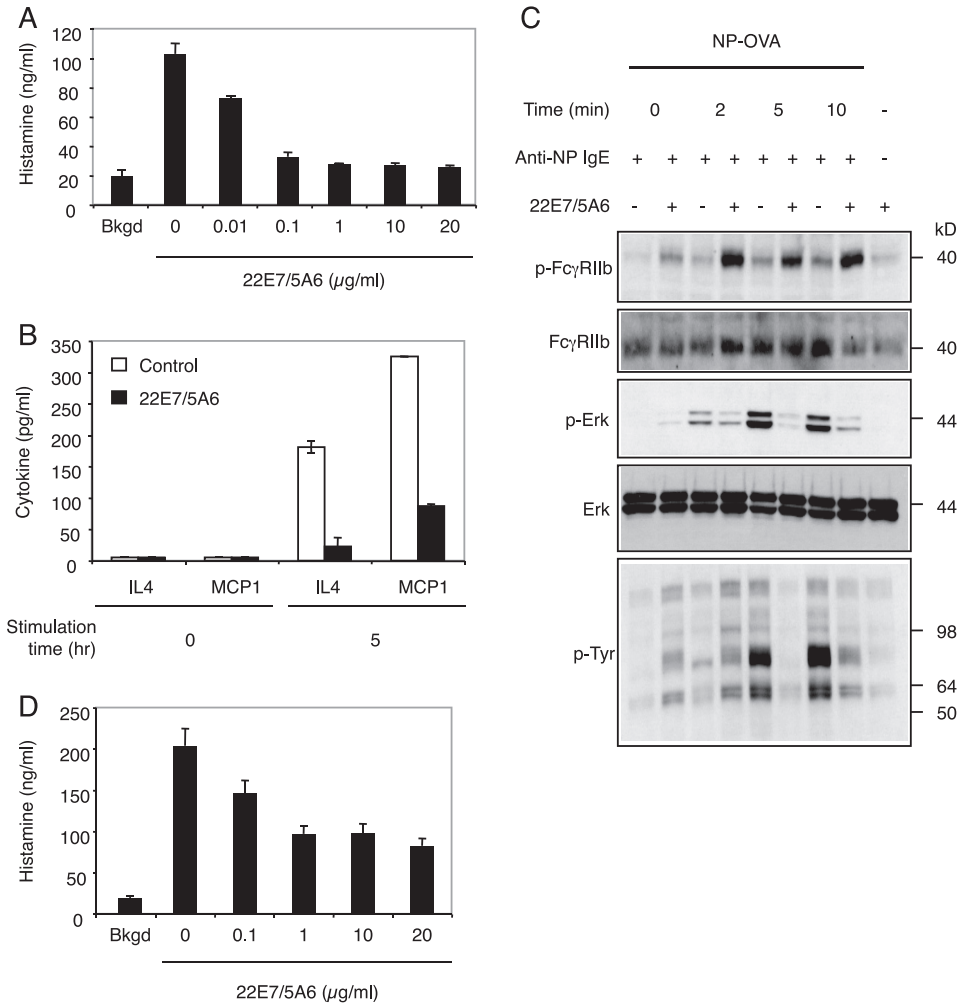


FIGURE 3. **22E7/5A6 bispecific antibody inhibits mast cell and basophil activation.** *A*, shown is the effect of 22E7/5A6 bispecific antibody on histamine release from RBL cells transfected with human FcεRIα and human FcγRIIb and activated through human FcεRI. *B*, shown is the effect of 22E7/5A6 bispecific antibody on interleukin-4 (*IL4*) and MCP-1 release from RBL cells transfected with human FcεRIα and human FcγRIIb and activated through human FcεRI. *C*, shown is a Western blot analysis of the effect of 22E7/5A6 bispecific antibody on phosphorylation of human FcγRIIb, ERK, and other protein tyrosines upon NP-ovalbumin (*NP-Ova*)-induced activation of NP-specific human IgE-sensitized RBL cells expressing human FcεRIα and human FcγRIIb. *D*, shown is the effect of 22E7/5A6 bispecific antibody on histamine release from primary human basophils stimulated with polyclonal anti-IgE. *Bkgd*, background; *MCP1*, monocyte chemotactic protein 1.

The 22E7/5A6 bispecific antibody inhibits degranulation, eicosanoid production, and cytokine production from RBL cell lines transfected with human FcεRIα and FcγRIIb (Fig. 3, *A* and *B*, and supplemental Fig. 2, *A* and *B*). The activity of 22E7/5A6 is due to bispecific antibody cross-linking of FcεRI and FcγRIIb because neither 22E7 by itself, 5A6 by itself, nor a combination of 22E7 and 5A6 together is able to inhibit RBL activation (supplemental Fig. 2*C*). Moreover, biochemical analysis of intracellular protein phosphorylation indicates that 22E7/5A6 treatment of RBL transfectants delivers an inhibitory signal to block FcεRI activation, as we observed the phosphorylation of FcγRIIb and the inhibition of protein phosphorylation events downstream of FcεRI activation upon 22E7/5A6 treatment (Fig. 3*C*). 22E7/5A6 also inhibits the activation of primary human peripheral blood basophils stimulated with polyclonal anti-IgE antibodies from six donors by 67 ± 9%, as assessed by histamine release (Fig. 3*D*).

A Chimeric Anti-human FcεRIα/Anti-mouse FcγRIIb Surrogate Bispecific Antibody Inhibits IgE-mediated Activation of Mast Cells in Vivo—22E7 and 5A6 are specific for human proteins and do not cross-react with the orthologous mouse proteins. Therefore, to assess the *in vivo* activity of a knobs-into-holes anti-FcεRIα/anti-FcγRIIb bispecific antibody, we created a chimeric mouse/human bispecific antibody surrogate for 22E7/5A6 consisting of the Fabs of 22E7 and a mouse anti-mouse FcγRIIb antibody 14H6 (supplemental Fig. 3*A*) combined with a human knobs-into-holes IgG1 Fc. In addition, we created and used mice transgenic for human FcεRIα (supplemental Fig. 4, *A*–*C*). These mice express human FcεRIα and mouse FcγRIIb, the targets of 22E7 and 14H6. When administered after the sensitization of cutaneous mast cells with antigen-specific IgE, the 22E7/14H6 bispecific antibody, which has comparable *in vitro* activity as 22E7/5A6 on RBL cell transfectants (supplemental Fig. 3*B*), inhibits mast cell activation in a PCA reaction in a dose-dependent manner, with near-complete suppression of mast cell activation upon treatment with 500 μg of bispecific antibody per mouse (Fig. 4, *A* and *B*).

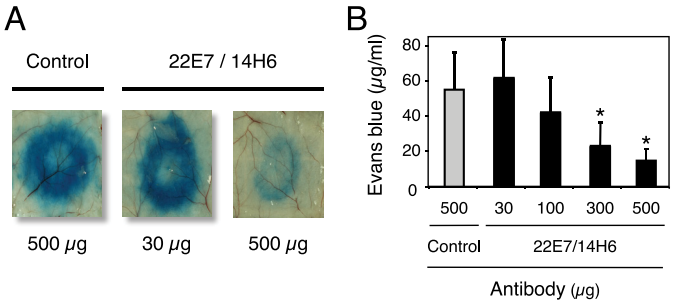
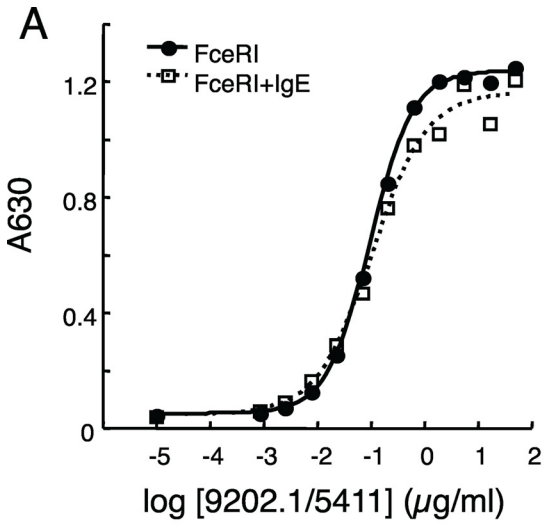
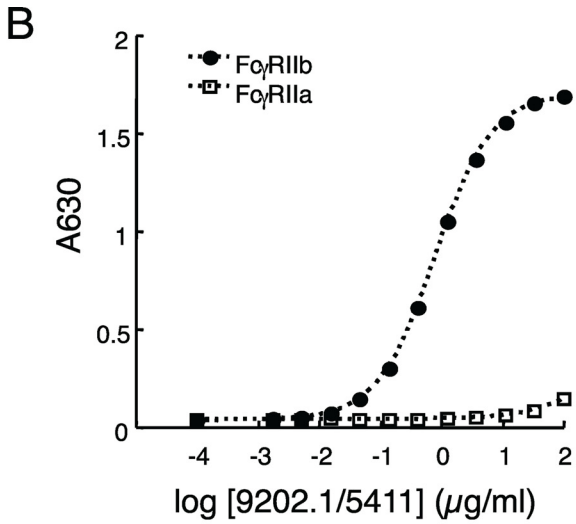
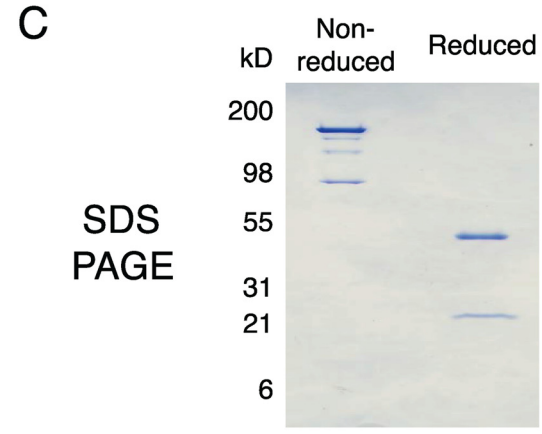


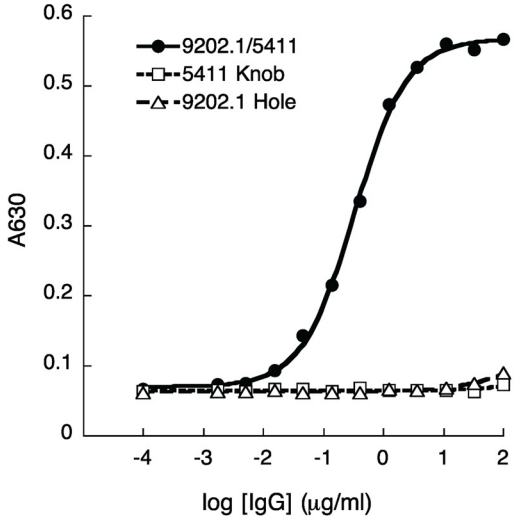
FIGURE 4. **22E7/14H6 bispecific antibody inhibits *PCA*.** Dermal mast cells of human FcεRIα transgenic mice were passively sensitized with NP-specific human IgE 24 h before activation. 22E7/14H6 bispecific antibody was administered after cutaneous mast cell sensitization, 2 h before mast cell activation. Mast cells were activated by intravenous injection of 200 μg NP-ovalbumin with 1% Evans blue. *A*, shown are pictures of skin from human FcεRIα transgenic mice treated with control or 22E7/14H6 bispecific antibody and subjected to PCA. *B*, shown is quantitation of Evans blue dye extracted from the flank skin of human FcεRIα transgenic mice treated with 22E7/14H6 antibody and subjected to PCA. *, $p < 0.05$, compared with control antibody treatment.

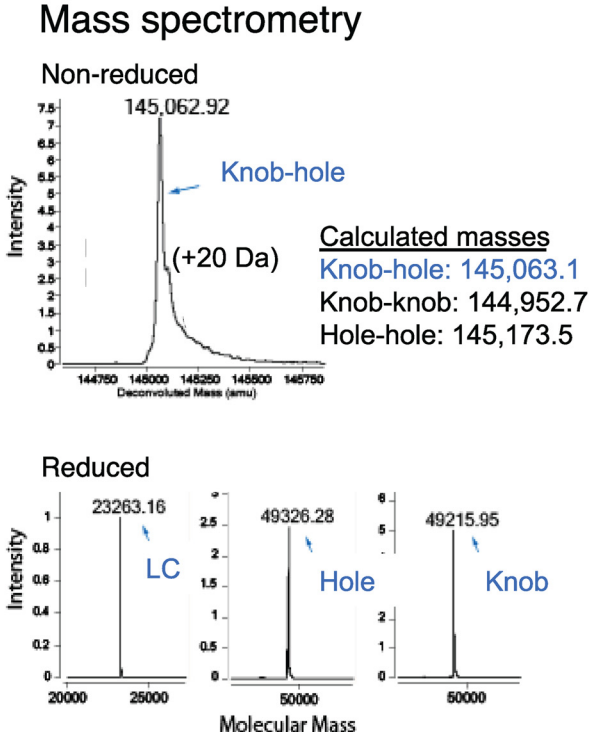
Generation of a Human Anti-human FcεRIα/Anti-human FcγRIIb Bispecific Antibody That Is Suitable as a Human Therapeutic—To identify a bispecific antibody that could be developed as a human therapeutic, we applied a phage display

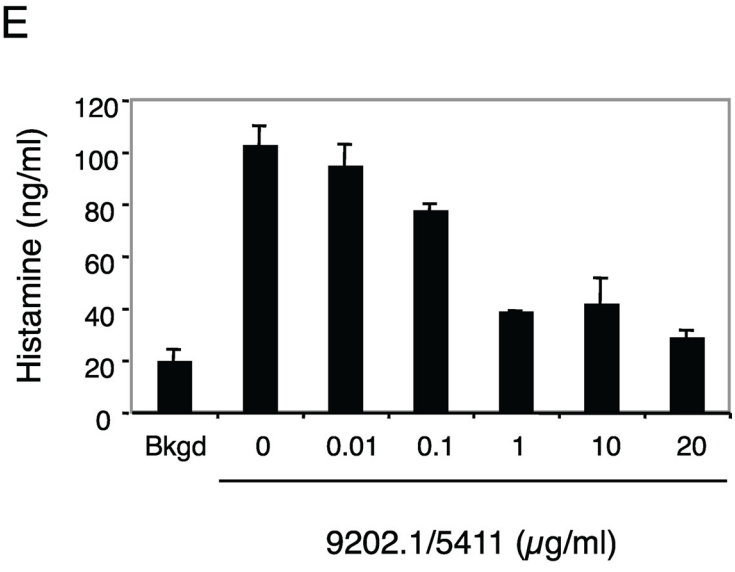

A
FceRI
FceRI+IgE
A630
log [9202.1/5411] (μg/ml)


B
FcγRIIb
FcγRIIa
A630
log [9202.1/5411] (μg/ml)


C
SDS PAGE
kD
Non-reduced
Reduced
200
98
55
31
21
6


D
9202.1/5411
5411 Knob
9202.1 Hole
A630
log [IgG] (μg/ml)


Mass spectrometry
Non-reduced
145,062.92
Knob-hole
(+20 Da)
Intensity
Calculated masses
Knob-hole: 145,063.1
Knob-knob: 144,952.7
Hole-hole: 145,173.5
Reduced
23263.16
LC
49326.28
Hole
49215.95
Knob
Molecular Mass


E
Histamine (ng/ml)
Bkgd
0
0.01
0.1
1
10
20
9202.1/5411 (μg/ml)

approach to generate a fully human IgG1 bispecific antibody targeting human FcεRIα and FcγRIIb. To enable the production of the bispecific antibody in a single cell, we identified Fabs against FcεRIα and FcγRIIb that share a single common light chain, thereby precluding the formation of mispaired heavy chain/light chain combinations during the expression and purification of the bispecific antibody. Antibodies with identical light chains that bind to human FcεRIα and FcγRIIb were identified from Fab phage libraries containing a single light chain sequence, with synthetic diversity introduced only at solvent-exposed positions within the heavy chain CDRs. We utilized an arbitrary light chain sequence based on a consensus κI sequence. Although we do not know whether the light chain contributes to our antibody binding affinities and/or specificities, it has previously been shown that high affinity antibodies can be generated from phage antibody libraries that have diversities only in the heavy chain CDRs (4). We did not humanize 22E7 and 5A6 because engineering of a common light chain for humanized 22E7 and 5A6 was not feasible. We identified a clone 9202.1, which binds to human FcεRIα with high affinity (K_d = 0.60 nM; supplemental Fig. 5*A*) and is not blocked by IgE binding to FcεRIα (Fig. 5*A*). We also identified a clone 5411 that binds to human FcγRIIb with high affinity (K_d = 35 nM; supplemental Fig. 5*B*) and specificity, as 5411 does not cross-react with human FcγRIIa (Fig. 5*B*). Clones 9202.1 and 5411 have distinct heavy chain sequences but the same light chain sequence.

We were able to produce the 9202.1/5411 bispecific antibody in *E. coli* cells with high yield and purity, as determined by gel electrophoresis, mass spectrometry, and ELISA binding assays (Fig. 5, *C* and *D*), by utilizing a single plasmid containing the knobs and holes heavy chains and the common light chain. Significantly, a 10-liter fermentation of 9202.1/5411 produced titers of ~350 mg/liter of antibody, a level that is well within the range of initial titers that can be scaled up to commercially viable expression levels. This yield is slightly higher than the titers reported previously for the expression of a conventional monospecific human IgG1 antibody in *E. coli* cells (29), probably due to the heavy chain knobs and hole mutations, which generally result in improved expression titers. Analysis of the purified intact 9202.1/5411 antibody by mass spectrometry shows a peak having the mass expected for the heterodimeric bispecific antibody and an adduct species of 20 daltons greater mass but no discrete peaks corresponding to knob/knob or hole/hole homodimers (Fig. 5*C*). In addition, the mass/charge envelope for the intact purified bispecific antibody contained spectra for only a single species with no evidence of homodimers (data not shown). The mass spectrometry data in conjunction with SDS-PAGE analysis of the purified non-reduced bispecific antibody, which shows a major band of the expected mass and several faint lower molecular weight protein bands in the 75–125-kDa range that likely represent incomplete oxidation of the parental bispecific antibody (Fig. 5*C*), indicate that the purified bispecific antibody does not contain measurable homodimeric species by these methods. The purified 9202.1/5411 bispecific antibody inhibits IgE-mediated activation of RBL cells transfected with human FceRIa and FcgRIIb, comparable with the 22E7/5A6 bispecific antibody (Fig. 5*E*).

TABLE 1

Pharmacokinetic parameters in SCID beige mice of 9202.1/5411 knobs-into-holes bispecific antibody expressed in *E. coli* bacteria and parental 9202.1 and 5411 antibodies expressed in mammalian Chinese hamster ovary (CHO) cells

Numbers are the average for the group (with three animals per group per time point). $AUC_{0\text{-last}}$, area under the plasma-concentration *versus* time curve; CL, clearance; $Half\text{-}life_{beta}$, elimination half-life; C_{max}, maximum concentration reached.

	9202.1/5411 bispecific antibody (*E. coli*)	9202.1 parental antibody (CHO)	5411 parental antibody (CHO)
$AUC_{0\text{-last}}$ (day·μg/ml)	1380	1240	1850
CL_{obs} (ml/day/kg)	5.70	5.88	3.34
$Half\text{-}life_{beta}$ (days)	12.1	13.5	21.2
C_{max} (μg/ml)	252	223	236

We assessed the pharmacokinetic properties of the human 9202.1/5411 IgG1 bispecific antibody in SCID beige mice and compared them to the pharmacokinetic properties of the parental 9202.1 and 5411 human IgG1 antibodies made in mammalian Chinese hamster ovary cells. Upon intravenous dosing, the bispecific antibody exhibited a biphasic pharmacokinetic profile with a distribution phase followed by an elimination phase, which is similar to the pharmacokinetic profile of a conventional IgG1 antibody (supplemental Fig. 6). Significantly, the serum concentrations and pharmacokinetic parameters calculated for the 9202.1/5411 bispecific antibody are similar to those obtained for the parental antibodies (Table 1), indicating that the bispecific antibody has pharmacokinetic properties comparable with conventional IgG produced in mammalian cells.

DISCUSSION

Bispecific antibodies have numerous potential clinical applications, including neutralization of different pathogenic mediators, redirection of cellular activity by recruitment of different cell types to each other, and modification of cell signaling by recruitment of two different receptors on the same cell. However, the development of bispecific antibodies as human therapeutics, especially for chronic administration in human diseases, requires a technology platform that exhibits high stability, favorable *in vivo* pharmacokinetic properties and fea-

FIGURE 5. **Generation and characterization of 9202.1/5411 bispecific antibody.** *A*, shown is an ELISA analysis of 9202.1/5411 bispecific antibody binding to recombinant human FcεRIα extracellular domain protein in the presence and absence of human IgE. *B*, shown is an ELISA analysis of 9202.1/5411 bispecific antibody binding to recombinant human FcγRIIb and FcγRIIa extracellular domains. *C*, shown is SDS-PAGE analysis of 9202.1/5411 bispecific antibody under non-reducing and reducing conditions indicates high purity and the expected masses of heavy and light chains (*LC*). Mass spectrometry analysis of 9202.1/5411 bispecific antibody (non-reduced and reduced) indicates expected masses of the intact bispecific antibody, common light chain, 9202.1 hole, and 5411 knob. *D*, shown is an ELISA analysis of 9202.1/5411 bispecific antibody, 5411 knob, and 9202.1 hole binding to both human FcεRIα and human FcγRIIb extracellular domains. Recombinant human FcγRIIb is directly coated on the ELISA plate followed by incubation with 9202.1/5411, 9202.1, or 5411. Bispecific binding is detected by further incubation with recombinant human FcεRIα extracellular domain followed by incubation with biotinylated human IgE binding and detection of the entire antibody/extracellular domain/IgE complex with HRP-conjugated streptavidin. *E*, shown is the effect of 9202.1/5411 bispecific antibody on histamine release from RBL cells transfected with human FcεRIα and human FcγRIIb and activated through human FcεRI. *Bkgd*, background.

sibility for large-scale manufacturing, properties that have been difficult to achieve to date (7–9).

In this study we have extended previous work (28) by developing a two-part antibody discovery strategy that facilitates proof-of-concept studies and clinical candidate antibody generation. Significantly, the adaptation of bacterial antibody production technology to the generation of knobs-into-holes, common light chain bispecific antibodies, enables the large-scale manufacturing of clinical candidates. Because the expression scheme utilizes *E. coli* bacteria, the resulting antibodies are not glycosylated and, therefore, lack Fc effector function (29, 36–38). This limits the applications of bispecific antibodies that are produced using this approach to therapeutic applications that do not require Fc effector functions but in some cases may enable the application of bispecific antibodies to targets where Fc effector function is not desired. Interestingly, a recent report describes Fc variants of aglycosylated antibodies with tight FcγRI binding, which could be adapted to our bispecific antibody system to enable the generation of bispecific antibodies with Fc effector functions (39). Aglycosylated antibodies have previously been shown to have normal FcRn binding and pharmacokinetics (29), consistent with our bispecific antibody pharmacokinetic data, and have not been immunogenic when administered to humans (40). Additional bispecific antibody technologies that are amenable to expression in mammalian cells, including the recently published dual-variable-domain immunoglobulin (10) and dual-acting-Fab immunoglobulin (41), provide alternative technological approaches to the generation of therapeutic bispecific antibodies that may be suitable for chronic dosing in human diseases. However, neither of these alternative formats is adaptable to the specific cross-linking of cellular receptors, and therefore, they cannot be applied to our therapeutic application of targeting FcεRIα and FcγRIIb, as discussed below.

We have applied our bispecific antibody technology to generate bispecific antibodies against FcεRIα and FcγRIIb that can inhibit IgE-induced activation of mast cells and basophils *in vitro* and *in vivo*. Importantly, targeting of IgE signaling through FcεRI is a clinically validated and efficacious approach for the treatment of asthma (13–15), but to date the successful generation of human bispecific agents targeting this pathway and suitable for the chronic treatment of human asthma patients has not been achieved. Two recent studies have reported the generation of bifunctional FcεRI cross-linkers that inhibit FcεRI activation *in vitro* and *in vivo* (25, 26). These cross-linkers are fusion proteins that consist of the Fc region of human IgG1 linked to either the Fc region of human IgE or an allergen protein and that thereby recruit FcγRIIb to either FcεRI or FcεRI that has been loaded with allergen-specific IgE, respectively. However, these bifunctional cross-linkers as well as all other agents developed to date that cross-link FcεRI with FcγRIIb or another inhibitory receptor have been limited by one or more liabilities that prohibit successful clinical development. In addition, neither the dual-variable-domain nor the dual-acting-Fab immunoglobulin formats is suitable for inhibiting FcεRI signaling, as these antibody formats may cross-link FcεRI to itself in the absence of FcγRIIb engagement, thereby leading to the activation of FcεRI and subsequent unacceptable anaphylactic reactions. In particular, a dual-acting-Fab antibody can bind either two of the same antigen or two different antigens such that some dual-acting-Fab antibodies against FcεRI and FcγRIIb could bind and cross-link two FcεRI, leading to receptor activation. Dual-variable-domain antibodies may not efficiently co-engage all FcεRI and FcγRIIb due to steric constraints associated with the tandem variable domain format, leading to some cross-linking and activation of FcεRI in the absence of FcγRIIb engagement. Our fully human bispecific antibody 9202.1/5411 is not subject to the limitations of these other cross-linking approaches. Specifically, the 9202.1/5411 bispecific antibody comprises an anti-FcεRIα Fab that is not blocked by IgE binding to FcεRIα, an anti-FcγRIIb Fab that does not cross-react with FcγRIIa or any other activating Fcγ receptor isoforms, and a fully human antibody format that has comparable pharmacokinetic properties to regular human IgG1 antibodies. Moreover, the 9202.1/5411 bispecific antibody can be expressed and purified at yields and purity levels that are compatible with large-scale manufacturing.

Our studies demonstrate that bispecific antibodies utilizing the knobs-into-holes, common light chain antibody format can be produced in large quantities in bacterial cells. Our bacterially produced bispecific antibodies are active *in vivo* and have pharmacokinetic properties that are comparable with those of regular human IgG1 antibodies expressed in mammalian cells. In addition, the bispecific antibody platform is fully human, such that human immunogenicity is minimized. Interestingly, the 9202.1/5411 bispecific antibody can bind FcεRIα with subnanomolar affinity and can discriminate between FcγRII homologs that have greater than 90% sequence identity despite having a single common light chain sequence. This is consistent with previous work demonstrating that the knobs-into-holes, common light chain antibody format can be used to generate antibodies that bind to a diverse range of antigens with high affinity and specificity (28). Thus, the bispecific antibody technology developed here may be generally applicable for the creation of bispecific therapeutic agents for human diseases.

Acknowledgments—We thank Meijuan Zhou and Jean Shu for assistance with in vivo studies, Bryan Irving for help in identifying and characterizing the 14H6 antibody, Andres Paler Martinez for help with Luminex studies, and Susan MacDonald for advice on human basophil isolation and stimulation.

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