A Recombinant Ig Fragment (IgCw-γεκ) Comprising the Cγ₁-Cε₂₋₄ and Cκ Domains Is an Alternative Reagent to Human IgE

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Human IgE is useful for immunological assays, such as sensitization of FcεRI-positive cells and IgE measurement. In this study, we report the development of a recombinant Ig fragment, designated IgCw-γεκ, as an alternative reagent to human IgE. IgCw-γεκ (~130 kDa) comprises two hybrid constant H chain regions (Cγ₁-Cε₂₋₄, each ~53 kDa) and two constant κ L chains (Cκ, each ~12 kDa) and lacks a V domain. The presence of Cγ₁ instead of Cε₁ within the H chain increased the production yield and facilitated assembly of the H and L chains. IgCw-γεκ was produced in cultured human embryonic kidney 293F cells, with a yield of ~27 mg/l. IgCw-γεκ bound to human FcεRIαRs expressed on the surface of rat basophilic leukemia-2H3 cells. A β-hexosaminidase release assay revealed that the biological activity of IgCw-γεκ was comparable with that of IgE. The IgE concentration measured using IgCw-γεκ as a standard was similar to that measured using IgE as a standard. These results suggest that the IgCw-γεκ molecule retains the basic characteristics of IgE, but does not cross-react with Ags, making it an alternative to the IgE isotype references used in a variety of immunological assays. *The Journal of Immunology*, 2022, 208: 772–779.

Human IgE is an Ab isotype that plays a role in allergic reactions and immunity against parasites (1). Most IgE Abs are generated by plasma cells in MALT in response to allergens; these Abs bind to the high-affinity IgE receptor (FcεRI) expressed by mast cells in tissues and by basophils in the blood. Binding of Ags to cell-bound IgE results in multivalent cross-linking of FcεRIs on mast cells and basophils, thereby triggering these cells to release chemical mediators stored inside granules; these mediators can trigger allergic manifestations, such as urticaria, rhinitis and asthma, swelling of the bowel, or systemic anaphylaxis (2).

IgE is present at extremely low levels in the blood or extracellular fluid of healthy humans. The total IgE concentration in serum is normally <1 μg/ml (0.12–0.3 μg/ml), which is several logs (10,000–50,000-fold) lower than that of IgG (5–10 mg/ml) (3). Even in highly allergic individuals, the concentration of serum IgE (sIgE) is still 1000-fold lower than that of serum IgG (4). Thus, IgE levels are often expressed in terms of kU/l (equivalent to IU/ml: 1 kU/l = 2.4 ng/ml IgE) (5). Elevated sIgE levels are often associated with severe allergic reactions, parasitic infections, hyper-IgE immunodeficiency syndrome (6), and an extremely rare IgE-producing myeloma (7). Measurement of sIgE is useful for diagnosis and/or management of atopic diseases and hyper-IgE immunodeficiency syndromes, as well as for appropriate dosing of patients undergoing anti-IgE therapy with omalizumab (8).

Assays designed to measure human IgE require a reference IgE as a standard; similarly, in vitro experiments designed to validate certain effects mediated by Ag-specific IgE Abs require IgE as an irrelevant isotype control. At present, most commercially available purified human IgE proteins recommended for use in quantitative and qualitative IgE assays are monoclonal and are prepared in one of three ways: 1) Abs are purified from the plasma of a myeloma patient with elevated IgE levels (e.g., products from Abcam, Athens Research & Technology, MyBioSource, Fitzgerald Industries International, Molecular Innovations, and Merck Millipore); in this case, IgE is produced by a single clone of a plasma cell, although none of the available myeloma-driven IgEs has known antigenic specificity; 2) Abs are produced in vitro by a monoclonal B cell hybridoma originated from a healthy donor and then purified (e.g., products from Abcam, Bioporto, Enzo Life Sciences, Diatec Monoclonals, Thermo Fisher Scientific, antibodies-online, and Abbiotec); or 3) Abs are produced by a human cell line that expresses recombinant human IgE and then purified (e.g., products from Bio-Rad). There is a possibility that these IgE Abs may show undesirable cross-reactions during IgE analysis, regardless of their antigenic specificity; cross-reactivity arises from the Ag-binding activity of the V domains (V_H and V_L) within the H and L chains, and the specificity of an Ab cannot be tested against all possible Ags.

In this study, we developed an alternative to human IgE reference standards. We generated a novel rIg fragment, designated IgCw-γ₁ε₂₋₄/κ (IgCw-γεκ for short), with a molecular mass of ~130 kDa. IgCw-γεκ comprises two hybrid constant H chains (Cγ₁-Cε₂₋₄) and

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two constant κ L chains (C_κ) and lacks a V domain. We show that IgCw-γεκ can be produced at a high yield by culture in human embryonic kidney (HEK) 293F cells, and that it can be used as an alternative to full-size IgE as a reference standard in quantitative and qualitative assays.

Materials and Methods

Plasmid construction

The DNA fragment encoding human C_ε (C_ε1-C_ε2-C_ε3-C_ε4) was cloned into the expression vector KV10-IgCw-γκ (9) between the *Nhe*I and *BamH*I restriction sites, thereby generating vector KV10-IgCw-εκ that contains human C_ε1−4 and human C_κ genes under the control of two individual CMV promoters (P_{CMV}) that allow simultaneous expression of the C_ε and C_κ chains with a leader sequence. Then, the DNA fragment encoding human C_γ1-hinge-C_ε2-C_ε3-C_ε4 was cloned into the KV10-IgCw-γκ vector between restriction sites *Nhe*I and *BamH*I, thereby generating vector KV10-IgCw-γεκ. The nucleotide and amino acid sequences of the H and L chains of IgCw-γεκ are shown in Supplemental Fig. 1. The KV10 plasmid was designed as a cassette vector to facilitate individual cloning of all types of Ab fragment genes (V_H, V_L, C_H, and C_L), along with upstream leader sequences, into specific sites. Plasmid vectors that express chimeric 6C407 IgE and 3D8 IgE, which have mouse-derived V domains and human-derived C domains (C_ε and C_κ), were constructed by replacing the C_γ1−3 gene with the C_ε1−4 gene in the pre-existing KV12-6C407 IgG and KV12-3D8 IgG vectors; the C_ε1−4 gene was inserted between restriction sites *Nhe*I and *Hind*III, thereby generating KV12-6C407 IgE and KV12-3D8 IgE. The cloning strategy used to construct the vectors is shown in Supplemental Fig. 2. The 6C407 and 3D8 Abs are specific for KIFC1 and DNA Ag, respectively (10). The only differences between KV10 and KV12 are the promoter type ($P_{EF1\alpha}$ and P_{CMV} in KV12) and restriction enzyme sites.

Preparation of Ig proteins

FreeStyle HEK293F cells (Thermo Fisher, catalog no. R79007), adapted to suspension culture in serum-free medium, was used for Ig production. Cells (1 × 10⁶ cells/ml in 100 ml) were seeded in a 500-ml flask (Corning, catalog no. 431145) 24 h prior to transfection to ensure that they reached the appropriate density (2 × 10⁶ cells/ml) at the time of transfection. Cells were cultured in serum-free FreeStyle 293 medium (Invitrogen, catalog no. 12338) under 8% CO_2 at 37°C, with orbital shaking at 130 rpm. Plasmids KV10 (encoding IgCw-εκ, IgCw-εγκ, 6C407 IgE, 6C407 IgG, and 3D8 IgE) were transiently transfected into HEK293F cells in 100 ml of FreeStyle medium using polyethylenimine (PEI) (Polyscience, catalog no. 23966-2). Briefly, PEI reagent (400 µg) was incubated with plasmid DNA (200 µg) at room temperature (RT) for 10 min and then inoculated onto 100 ml of cells to achieve a final PEI concentration of 4 µg/ml. After 7 d, the culture supernatants were harvested by centrifugation. After clarifying the supernatants by passage through a 0.45-µm cellulose acetate filter, the supernatants were subjected to affinity chromatography using CaptureSelect KappaXP-Agarose (Thermo Fisher Scientific, catalog no. 2943212005), which captures the C_κ domain. Purification of IgCw-γεκ was also performed using CaptureSelect IgG-C_H1 agarose (Thermo Fisher Scientific, catalog no. 194320005). The concentration of the purified Ig proteins was determined using the following formula: protein concentration (mg/ml) = (absorbance at 280 nm × molecular mass × dilution factor)/extinction coefficient (ε) at 280 nm; ε was calculated from the amino acid sequence (http://web.expasy.org/protparam/). Polyclonal human IgE was purchased from Sigma-Aldrich (catalog no. I8640).

Immunoblot analysis

The 7-d culture supernatants from transfected HEK293F cells were resolved by reducing and nonreducing SDS-PAGE and transferred to nitrocellulose membranes. To detect human ε H chain, the membrane was probed with primary mouse anti-human IgE (Sigma, catalog no. I6510) and secondary horse anti-mouse IgG-HRP (Cell Signaling Technology, catalog no. 7076) Abs. To detect human κ L chain, the membrane was probed with primary goat anti-human κ-chain (Thermo Fisher Scientific, catalog no. 31129) and secondary rabbit anti-goat IgG-HRP (Thermo Fisher Scientific, catalog no. 81-1620) Abs. Immunoreactive protein was visualized using an ECL Kit (GE Healthcare, catalog no. RPN2106).

Size-exclusion chromatography

The integrity and purity of the purified IgCw-γεκ and 6C407 IgE was analyzed by size-exclusion chromatography (SEC) using a Shimadzu UFLC System (DGU-20A3) fitted with a TSKgel G3000SW$_{XL}$ size-exclusion column (30 cm × 7.8 mm; Toso Haas). Proteins (1 mg/ml in 30 µl PBS) were

injected onto the column and run in the mobile phase of 100 mM HEPES/85 mM HNaSO4 (pH 6.8) at a flow rate of 1 ml/min. Chromatograms were obtained by monitoring the absorbance at 280 nm.

Rat basophilic leukemia-2H3 cell culture

The rat basophilic leukemia (RBL)-2H3 cell line (ATCC number CRL-2256) was maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 5% CO_2 incubator at 37°C.

Establishment of human FcεRIα-expressing RBL-2H3 cells

DNA encoding human FcεRIα (hFcεRIα) was cloned into pCDH-CMV-MCS-EF1-Puro vectors (System Biosciences, catalog no. CD510B-1). Lentivirus particles were generated by cotransfecting 293T cells (2 × 10⁶ cells) with 4 µg of pCDH-CMV-hFcεRIα, 3 µg of GAG-pol, and 1 µg of pVSV-G in a tube containing 16 µg of polyethylenimine. Culture medium containing the recombinant lentivirus was harvested at 48 h posttransfection. RBL-2H3 cells were seeded (2 × 10⁶ cells/well) in 60-mm dishes overnight and incubated with fresh medium containing 10 µg/ml polybrene and 1 ml of the lentiviral supernatant. At 48 h postinfection, virus-containing medium was removed completely, and cells were selected for ~1 wk with 5 µg/ml puromycin to establish stable RBL-2H3-hFcεRIα cells.

Flow cytometry analysis

To detect expression of hFcεRIα-chain on the cell surface, RBL-2H3 cells and RBL-2H3-hFcεRIα cells (1 × 10⁶ cells) were trypsinized, washed with cold PBS, and fixed by incubation with 4% paraformaldehyde (PFA) in PBS for 10 min at RT. Then, cells were incubated for 1 h at 4°C with an allophycocyanin-conjugated mouse anti-human FcεRI Ab (Abcam, catalog no. 155369) diluted in buffer S (0.5% BSA and 2 mM EDTA prepared in PBS, pH 8.5). To detect binding of Ig proteins (IgCw-γεκ, 6C407 IgE, and polyclonal human IgE) to the cell surface, RBL-2H3 cells and RBL-2H3-hFcεRIα cells (1 × 10⁶) were treated for 3 h at 37°C with Ig proteins (final concentration, 1 µM). After washing three times with cold PBS, cells were fixed for 10 min at RT with 4% PFA in PBS. Then, cells were incubated with a primary goat anti-human IgE (ε-chain specific) Ab (Sigma-Aldrich, catalog no. I6284), followed by a secondary PE-conjugated donkey anti-goat IgG Ab (Abcam, catalog no. Ab7004). All Abs were diluted in buffer S. After washing three times with cold PBS, cells were resuspended in 4% PFA and analyzed by flow cytometry using a FACSCanto II cytometer (BD Biosciences).

β-hexosaminidase release assay

The β-hexosaminidase release assay was performed as previously described (11), with slight modifications. Briefly, RBL-2H3-hFcεRIα cells seeded in a 24-well plate (5 × 10⁵ cells/ml) were incubated overnight, sensitized with 10 nM of Ig protein (IgCw-γεκ, 6C407 IgE, polyclonal human IgE, or 6C407 IgG), and incubated for 3 h at 37°C under 5% CO_2. The cells were then washed twice with 500 µl of Siraganian buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 25 mM PIPES, 40 mM NaOH, 1 mM CaCl₂, and 0.1% BSA, pH 7.2). An aliquot (160 µl) of Siraganian buffer was added to the cells, and incubation was continued for 10 min at 37°C/5% CO_2. Goat anti-human IgG κ-chain (10 µg/ml), suspended in 20 µl of Siraganian buffer, was added to the cells for 20 min at 37°C to stimulate cell degranulation. The supernatants were transferred to 96-well plates (50 µL/well) and incubated with 50 µl of p-NAG substrate (1 mM para-nitrophenyl-*N*-acetyl-β-D-glucosaminide, Sigma-Aldrich; catalog no. N9376) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 200 µl of Stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance of the reaction solutions was measured in a microplate reader (Molecular Devices) at 405 nm. Controls without Ig protein were used to measure spontaneous release. Total β-hexosaminidase release was obtained by lysing cells with 0.1% Triton X-100 (Sigma-Aldrich) prior to supernatant removal. The percentage of released β-hexosaminidase was calculated using the following formula:

$$\% \ \beta\text{-hexosaminidase release} = (\text{absorbance of test samples}$$

$$- \text{absorbance of Siraganian buffer})/(\text{absorbance of total release}$$

$$- \text{absorbance of Siraganian buffer}).$$

To investigate the inhibitory effect of IgCw-γεκ on the activation of IgE-sensitized RBL-2H3-hFcεRIα cells, cells seeded in 24-well plates (5 × 10⁵ cells/ml) were incubated with a mixture of 6C407 IgE (10 nM) and IgCw-γεκ (two-fold dilutions starting from 20 nM) for 3 h at 37°C/5% CO_2, or sensitized by incubation with 6C407 IgE (10 nM) for 3 h prior to treatment with IgCw-γεκ (two-fold dilutions starting from 40 nM), or incubated with

IgCw-γεκ (10 nM) for 3 h prior to treatment with 6C407 IgE (two-fold dilutions starting from 40 nM) for 2 h at 37°C/5% CO_2. The cells were washed twice with 500 μl of Siraganian buffer, followed by incubation with 160 μl of Siraganian buffer for 10 min at 37°C in 5% CO_2. Cells were treated with 20 μl of a mixture of biotinylated Protein L (Thermo Fisher Scientific, catalog no. 29997; final concentration = 140 nM) and streptavidin-fluorescein (Vector Laboratories, catalog no. SA-5001; final concentration = 70 nM) for 20 min at 37°C. The supernatants were transferred to 96-well plates (50 μL/well) and incubated with 50 μl of p-NAG substrate at 37°C for 1 h. The subsequent procedures were performed as described above.

ELISA

The concentration of IgE in the samples was measured using an IgE Human Uncoated ELISA Kit (Thermo Fisher Scientific, catalog no. 88-50610-22). Briefly, the wells of a 96-well plate were coated overnight at 4°C with capture Ab (100 μl/well) in PBS, pH 7.4), washed three times with TBST (pH 7.4), and blocked with blocking buffer (PBS with 0.1% Tween 20, and 1% BSA; 250 μl/well) at RT for 2 h. Wells were incubated for 2 h at RT with IgE samples (3D8 IgE, 6C407 IgE, or human plasma [Sigma H4522]), and two-fold serial dilutions (from 250 ng/ml) of standard (polyclonal human IgE and IgCw-γεκ) in assay buffer (PBS with 0.05% Tween 20, and 0.5% BSA). Next, a detection Ab (HRP-conjugated anti-human IgE mAb; 100 μl/well) diluted with assay buffer was added to the wells for 1 h at RT. Each incubation step was followed by washing three times with TBST. Each well was incubated with 100 μl/well of substrate tetramethylbenzidine solution for 15 min at RT, followed by 100 μl/well of Stop solution (2N H_2SO_4). Absorbance at 450 nm was measured in a microplate reader. The concentrations of the IgE samples were determined by interpolating y-axis values from two different standard curves generated using known concentrations of polyclonal human IgE and IgCw-γεκ.

Results

Design rationale for IgCw-γεκ

The IgCw-γεκ molecule was based on a hybrid C_H form of C_γ1-C_ε2–4; this is because the C_γ1 domain plays a critical role in folding and assembly of IgG Abs via the endoplasmic reticulum (ER)-quality control system (ERQC) (12–14). After translocation into the ER, incompletely folded H chains are bound to the molecular chaperone BiP via the C_H1 (C_γ1) domain until they associate with folded L chains. The association of the unfolded C_H1 domain with the folded C_L domain of the L chain allows the C_γ1 chains to fold, leading to secretion of correctly assembled IgG. By contrast, the ER-quality control pathway of IgE is unknown.

Assembly and expression of the IgCw-ε form are improved by replacing C_ε1 with C_γ1

Ig molecules were produced by suspension culture of the HEK293F cells transiently transfected with plasmids encoding 6C407 IgE, IgCw-εκ, or IgCw-γεκ. The expected structures of the expressed proteins are shown in Fig. 1A. After 7 d of culture, we checked the assembly of IgCw-εκ and IgCw-γεκ secreted from HEK293F transfectants by immunoblotting under reducing and nonreducing conditions using Abs specific for the C_ε and C_κ chains of human Ig (Fig. 1B).

A positive control 6C407 IgE (predicted molecular mass of 190 kDa) showed H and L chains of the expected size under reducing conditions: bands between 60 – 140 kDa were detected by anti-C_ε, and a single band of ~25 kDa was detected by anti-C_κ. Under nonreducing conditions, a band at ~190 kDa was detected by anti-C_ε, whereas multiple bands, including a major band at ~190 kDa, were detected by anti-C_κ. The observation of bands <95 kDa detected by anti-C_ε under reducing conditions and a band of 190 kDa detected by anti-C_κ Ab under nonreducing conditions may be due to incomplete assembly or partial degradation (Fig. 1B, left panel). IgCw-εκ showed bands >53 kDa under reducing conditions and a band of 130 kDa under nonreducing conditions (Fig. 1B, middle panel). However, anti-C_κ detected a single protein band at ~12 kDa even under nonreducing conditions, indicating that covalent association between

C_ε and C_κ chains did not occur when expressed in cells. Thus, the band at >130 kDa detected by anti-C_ε under nonreducing conditions is likely an aggregated form of the C_ε chains that bind to anti-C_ε.

IgCw-γεκ yielded bands of predicted sizes (~53 kDa and ~12 kDa corresponding to the $C_{\gamma\varepsilon}$ and C_κ chains, respectively) under reducing conditions (Fig. 1B, right panel). Under nonreducing conditions, multiple and smeared bands between 50 and 240 kDa were observed with both anti-C_ε and anti-C_κ, suggesting that the covalent association of $C_{\gamma\varepsilon}$ and C_κ chains occurred simultaneously with partial degradation or incomplete assembly of $C_{\gamma\varepsilon}$ and C_κ, as seen for 6C407 IgE. Under nonreducing conditions, anti-C_κ detected a very small amount of free C_κ chains during IgCw-γεκ expression. Taken together, the data suggest that the H chain (C_γ1-hinge-C_ε2-C_ε3-C_ε4) of IgCw-γεκ can associate covalently with the L chain (C_κ) during protein expression, as observed for full-size IgE; this is in contrast to observations for the C_ε1–4 chain of IgCw-εκ.

*The yield and purity of IgCw-γεκ are comparable with those of a full-size IgE*

Next, we compared the yields of Ig proteins produced by suspension culture of HEK293F cells. On day 7 posttransfection, IgE and IgCw-εκ proteins were purified from culture medium using KappaXP-agarose, which binds to the human C_κ domain. IgCw-γεκ was purified using KappaXP-agarose and IgG-C_H1-agarose. SDS-PAGE analysis of the purified Ig proteins showed that IgE and IgCw-γεκ were of the expected sizes under nonreducing conditions (>190 and ~130 kDa, respectively). Under reducing conditions, two protein bands of the expected size were observed at ~70 kDa and 25 kDa for IgE and at ~53 kDa and 12 kDa for IgCw-γεκ. This indicates that IgCw-γεκ was generated as an H_2L_2 form, like full-size IgE (Fig. 2A). IgCw-εκ showed a single band corresponding to the C_κ protein under nonreducing and reducing conditions, indicating that only the C_κ protein was recovered due to a lack of covalent association between the C_ε and C_κ chains, which is consistent with the result in Fig. 1B. SEC confirmed correct assembly of the purified IgCw-γεκ (Fig. 2B, upper panel), with a major peak with an apparent molecular mass of 130 kDa corresponding to IgCw-γεκ, and a minor peak with a larger molecular mass (possible protein aggregates). This peak profile was similar to that observed for IgE (Fig. 2B, lower panel).

The average yield of IgCw-γεκ purified using KappaXP-agarose was ~17 mg/l, which is equivalent to ~17 mg/l of 6C407 IgE. The yield of IgCw-γεκ purified using IgG-C_H1-agarose was ~27 mg/l. The yield of IgCw-γεκ protein was 1.6-fold higher when purified using IgG-C_H1-agarose rather than KappaXP-agarose (Table I). The lower yield of IgCw-γεκ from the KappaXP-agarose resin is likely due to occupation of the resin by free C_κ chains, thereby decreasing the amount of IgCw-γεκ binding to the resin. In fact, when IgCw-γεκ was purified using KappaXP-agarose, which has affinity for the C_κ domain, a trace amount of free C_κ chains (~12 kDa) was detected in the eluted fraction (Supplemental Fig. 3A). C_κ chains were detected in the flow-through after affinity chromatography using IgG-CH1 agarose, but not in the flow-through from KappaXP-agarose (Supplemental Fig. 3B), indicating binding of free C_κ chains to KappaXP-agarose. The problem of lower IgCw-γεκ yield from KappaXP-agarose resin was resolved by using IgG-CH1-agarose, which has affinity for the C_γ1 domain; this means that free C_κ chains are washed out, leaving only fully assembled IgCw-γεκ. The yield of IgCw-εκ (in fact, only free C_κ chains) was ~1 mg/l, much lower than that of the other two proteins.

IgCw-γεκ is recognized by FcεRI

Next, we examined whether IgCw-γεκ binds to the high affinity receptor for IgE (FcεRI). The FcεRIα-chain is a transmembrane

FIGURE 1. Theoretical structures and expression of three Ig proteins (IgE, IgCw-εκ, and IgCw-γεκ). **(A)** Schematic representation of the three Ig molecules. IgE comprises two ε H chains (~67–70 kDa each) and two L chains (25 kDa each). The ε H chain has high carbohydrate content (12% by molecular mass) with seven potential N-glycosylation sites across the $C_\varepsilon 1$–3 domains, making the molecular mass of IgE ~190 kDa (28, 29). Predicted N-glycosylation sites in each C_ε domain are denoted by six closed circles and one open circle (complex and oligomannose glycans, respectively). The predicted molecular mass of the glycosylated IgCw-εκ and IgCw-γεκ is ~130 kDa, with two C_H (~53 kDa each) and two C_L (12 kDa each) chains. IgCw-γεκ has a hybrid H chain: $C_\gamma 1$-hinge-$C_\varepsilon 2$–4. **(B)** Western blotting of the three Ig proteins in 7-d culture supernatants from transfected HEK293F cells. Proteins were detected by anti-C_ε and anti-C_κ chain Abs under reducing and nonreducing conditions.
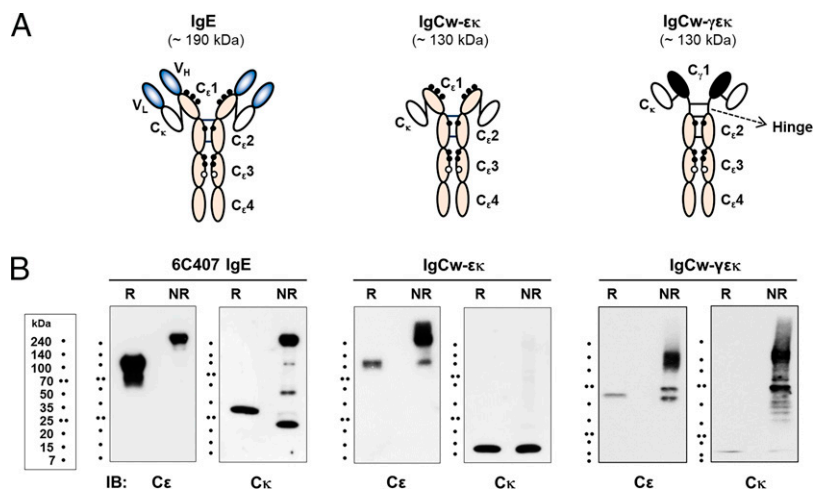
protein that associates with β and γ subunits to form the high-affinity IgER responsible for binding IgE-Fc. First, we established an RBL-2H3 cell line (an RBL mast cell model) transfected with the α subunit of human FcεRI (designated RBL-2H3-hFcεRIα cells). The hFcεRIα subunit can assemble with endogenous rat β and γ subunits, making it a functional substitute for the rat FcεRIα subunit (15). Expression of FcεRIα on the surface of RBL-2H3-hFcεRIα cells was confirmed by flow cytometry using an Ab specific for the FcεRIα-chain (Fig. 3A, 3B); the results showed a marked shift in the fluorescence intensity of RBL-2H3-hFcεRIα cells compared with that of parental RBL-2H3 cells. Next, we examined binding of IgCw-γεκ to RBL-2H3-hFcεRIα cells. The results showed that IgCw-γεκ and positive control IgEs (6C407 IgE and polyclonal human IgE) bound to the RBL-2H3-hFcεRIα cells in the same way as full-size IgE, but not to RBL-2H3 cells (Fig. 3C, 3D), suggesting that IgCw-γεκ is recognized by FcεRI.

IgCw-γεκ can be used as a reagent to monitor IgE-mediated reactions

Mast cell activation is triggered by cross-linking of IgE-bound FcεRI by Ags (or anti-IgE Abs), leading to the release of mediators from cytoplasmic granules (degranulation). Therefore, we examined activation of RBL-2H3-hFcεRIα via cross-linking of IgCw-γεκ–bound FcεRI by measuring the release of β-hexosaminidase. The results showed that cross-linking of IgCw-γεκ by anti-human C_κ triggered the release of β-hexosaminidase (Fig. 4A). As expected, positive controls (6C407 and polyclonal human IgE) triggered the release of β-hexosaminidase, whereas negative control 6C407 IgG did not (Fig. 4B).

Next, we investigated the inhibitory effect of IgCw-γεκ on activation of IgE-sensitized RBL-2H3-hFcεRIα by measuring the release of β-hexosaminidase. Selective cross-linking of IgE-bound FcεRI was achieved by treatment with a mixture of biotinylated protein L and streptavidin-fluorescein (Fig. 4C). The mixture of biotinylated protein L and streptavidin-fluorescein did not cross-link IgCw-γεκ–bound FcεRI because IgCw-γεκ lacking a V_κ domain does not interact with protein L. The inhibitory effect of IgCw-γεκ on β-hexosaminidase release by IgE-sensitized cells was concentration dependent, regardless of the fact that cells were coincubated with a mixture of 6C407 IgE and IgCw-γεκ (Fig. 4D), or sequentially with 6C407 IgE followed by IgCw-γεκ (Fig. 4E), or with IgCw-γεκ followed by 6C407 IgE (Fig. 4F). The release of β-hexosaminidase from IgE-sensitized cells was inhibited by 72% in the presence of a mixture comprising an equivalent molar concentration of 6C407 IgE and IgCw-γεκ, by 31% when cells preincubated with 6C407 IgE were treated with IgCw-γεκ, and by 63% when cells preincubated with IgCw-γεκ were treated with 6C407 IgE. These results support the notion that IgCw-γεκ can be used as a reagent in studies of IgE-mediated reactions.

IgCw-γεκ can be used as a reference for measuring IgE concentrations

Finally, we asked whether IgCw-γεκ can be an alternative to full-size IgE as a reference used to measure IgE concentrations. Two standard curves of samples containing known concentrations of human polyclonal IgE (Fig. 5A) and IgCw-γεκ (Fig. 5B) were generated by ELISA. Two monoclonal IgE samples (6C407 and 3D8) and

FIGURE 2. Analysis of the purity and integrity of purified proteins. **(A)** SDS-PAGE of the purified proteins on 4–20% SDS-PAGE gels, followed by Coomassie staining. The IgCw-εκ and 6C407 IgE proteins were purified using KappaXP-agarose, and IgCw-γεκ was purified using IgG-C_H1 agarose. **(B)** SEC analysis of purified IgCw-γεκ and 6C407 IgE.
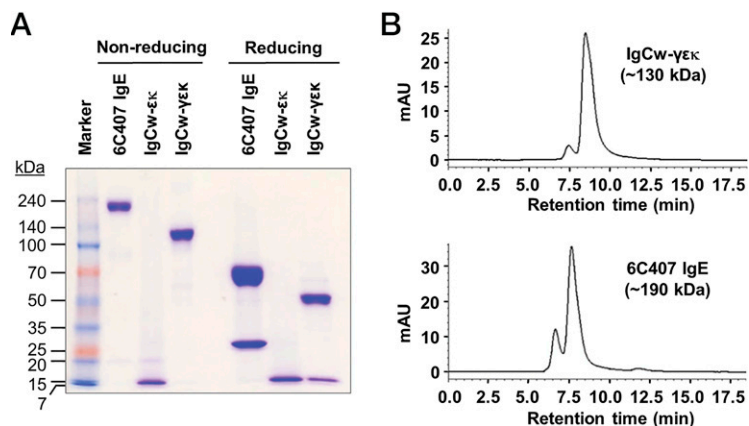
Table I. The yield of each Ig molecule purified from the supernatant of cultured HEK293F cells

Protein	Affinity Chromatography	Average Yield (mg/l)
6C407 IgE	CaptureSelect KappaXP-agarose	17.0
IgCw-εκ	CaptureSelect KappaXP-agarose	1.0
IgCw-γεκ	CaptureSelect KappaXP-agarose	17.0
	CaptureSelect IgG-C_H1-agarose	27.0

human plasma of unknown concentration were processed in the same manner. The concentration of these IgE samples was determined by interpolation of the respective curves (Table II) using the following linear interpolation formula: $x = x_1 + (x_2 - x_1) \times (y - y_1)/(y_2 - y_1)$ (https://formulas.tutorvista.com/math/interpolation-formula.html). The IgE concentrations determined from the standard curves for IgCw-γεκ were normalized by multiplying by the ratio of the molecular mass (molar ratio of IgE:IgCw-γεκ = 1.462:1). The normalized concentrations of 6C407 IgE and 3D8 IgE were 70.11 ng/ml and 23.01 ng/ml, respectively. These are almost equivalent to the concentrations (70.42 ng/ml for 6C407 IgE and 25.01 ng/ml for 3D8 IgE) determined from the standard curve constructed using polyclonal human IgE. Moreover, the concentration of plasma IgE determined from two standard curves was almost identical, 344.9 ng/ml and 346.9 ng/ml. These results suggest that IgCw-γεκ can be used as a reference molecule for measuring human IgE concentrations.

Discussion

To provide an alternative to human IgE, which is often produced with a low yield, we generated rIgCw-γεκ in HEK293F cells. The results showed that IgCw-γεκ is a valuable alternative to full-size human IgE for use in immunological research. The benefits of IgCw-γεκ are as follows 1): it is produced in a fully assembled form, with high yield, by culture in HEK293F cells; also, it is purified easily by affinity chromatography; 2) it can be used as a standard reagent in assays that measure IgE concentrations; 3) it is unlikely to react with any Ags because it lacks a V domain, making it useful as an isotype control for human IgE in bioassays; and 4) it is produced by a cell line cultured in serum-free medium optimized for the cells; therefore, the product is not contaminated by Abs of other isotypes or with bovine serum.

Previously, we produced an rIg fragment for use as an alternative to reference IgG isotypes; IgCw-γκ comprises the $C_γ1$–3 chain and $C_κ$ chain of human IgG and lacks V regions. The $C_γ1$–3 H and $C_κ$ L chains of IgCw-γκ were expressed as a covalently associated form (~98 kDa) in culture supernatants of HEK293F cells (9, 10). In contrast to IgCw-γκ, the $C_ε1$–4 and $C_κ$ chains of IgCw-εκ were secreted individually; only the $C_ε1$–4 H chains were associated covalently (Figs. 1B, 2A). A potential explanation for this difference is the ERQC, which allows secretion of only correctly assembled Ig molecules (14, 16). The ERQC is bypassed by some engineered H chains and under pathological conditions that cause H chain disease (17–19). The $C_γ1$–3 chain might be controlled by the ERQC, whereas the $C_ε1$–4 chain might evade it. In the ERQC, the $C_γ1$ domain of IgG controls assembly and secretion of IgG; however, nothing is known about the contribution made by the $C_ε1$ domain assembly of IgE Abs. The $C_γ1_ε2$–4 and $C_κ$ chains of the IgCw-γεκ molecule were secreted in a covalently associated form. Thus, replacement of the $C_ε1$ domain with the $C_γ1$ domain may direct assembly of the $C_γ1_ε2$–4 chain via the ERQC.

We successfully purified the IgCw-γεκ protein on a laboratory scale by affinity chromatography on KappaXP-agarose and IgG-C_H1-agarose; however, other purification methods will be cheaper and more effective if the IgCw-γεκ protein is to be purified on a large scale. Such methods include mixed-mode chromatography with MEP HyperCel (Pall Corporation), which has been used successfully to purify human IgE (20); thiophilic interaction chromatography,
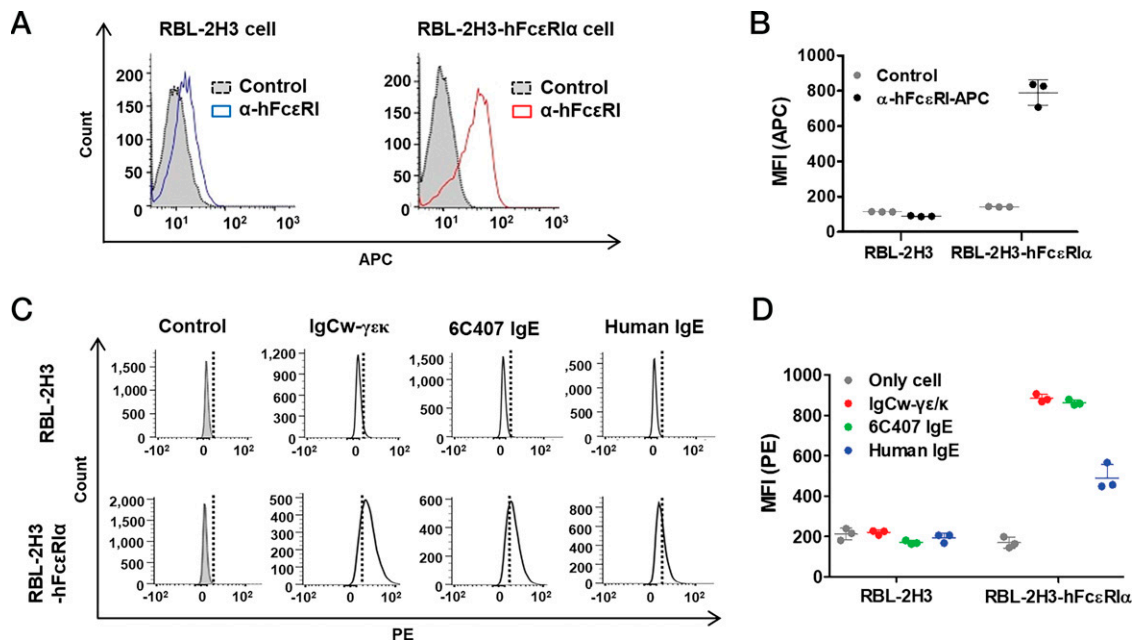

FIGURE 3. Flow cytometry analysis of hFcεRIα-chain expression on the cell surface and binding of Ig proteins to cells. (**A** and **B**) Cell surface expression of the hFcεRIα-chain on RBL-2H3 and RBL-2H3-hFcεRIα cells was examined using an allophycocyanin-conjugated anti-FcεRI Ab. (**C** and **D**) Binding of Ig proteins to RBL-2H3-hFcεRIα cells. Cells were treated with Ig proteins for 3 h at 37°C. Cell surface-bound Igs were detected using a primary goat anti-human IgE (ε-specific) Ab, followed by a secondary PE-conjugated donkey anti-goat IgG Ab. No protein indicates that the cells were treated with primary and secondary Abs alone. Representative cytometry profiles are shown (A and C). Data are presented as the mean fluorescence intensity ± SD of three independent experiments (B and D).
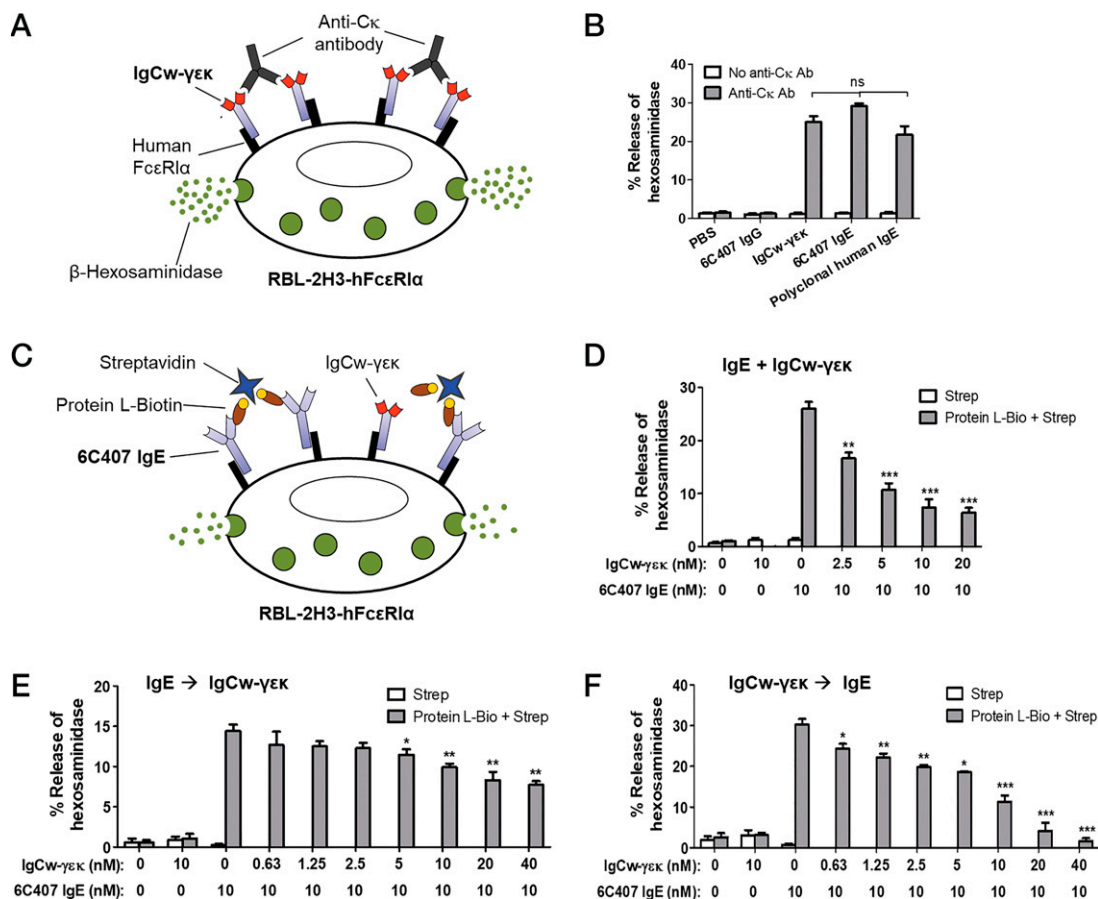
FIGURE 4. Effect of IgCw-γεκ on degranulation of RBL-2H3-hFcεRIα cells, detected by measuring extracellular β-hexosaminidase activity. (**A** and **B**) Effect of IgCw-γεκ on β-hexosaminidase release. RBL-2H3-hFcεRIα cells pretreated with Ig proteins (6C407 IgG, 6C407 IgE, polyclonal human IgE, or IgCw-γεκ) were incubated with a goat anti-human IgG κ-chain Ab. β-hexosaminidase release from the cells was measured. (**C–F**) Inhibitory effects of IgCw-γεκ on β-hexosaminidase release by IgE-sensitized RBL-2H3-hFcεRIα cells. (C) Schematic representation of the experimental design. Cells were coincubated with 6C407 IgE, along with various concentrations of IgCw-γεκ (D), or pretreated with 6C407 IgE prior to IgCw-γεκ (E), or pretreated with IgCw-γεκ prior to 6C407 IgE (F). Next, cells were stimulated with a mixture of biotinylated protein L and streptavidin-fluorescein. Left panels show a schematic representation of the experimental design. Data are expressed as the mean ± SE of three independent experiments (B and D). All *p* values were calculated using a two-tailed Student *t* test. Statistical significance is indicated on the graphs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

which has been used for mouse IgE purification and is capable of purifying any IgE, irrespective of species (21); and conventional protein purification methods, such as gel filtration, ion-exchange chromatography, or a combination of these processes. In addition, understanding the stability of the IgCw-γεκ protein would be very helpful when working with IgCw-γεκ as an alternative to human IgE. Protein stability usually infers resistance to unfolding, aggregation, and degradation by physical and chemical stresses, such as high temperature, lyophilization, organic cosolvents, denaturing reagents, and proteolytic enzymes.

In this study, we conducted SDS-PAGE to assess the integrity of the IgCw-γεκ protein after lyophilization and after storage in PBS buffer at 4°C for 8 wk. The results showed that, similar to IgE, the integrity of IgCw-γεκ was not affected at all by storage conditions or lyophilization (Supplemental Fig. 4).

IgCw-γεκ induced degranulation of RBL-2H3-hFcεRIα cells triggered by anti-Igκ; this process was almost as efficient as that triggered by IgEs (Fig. 4B), demonstrating that the structure of the Fc region (Cε2–4) of IgCw-γεκ is analogous to that of native human
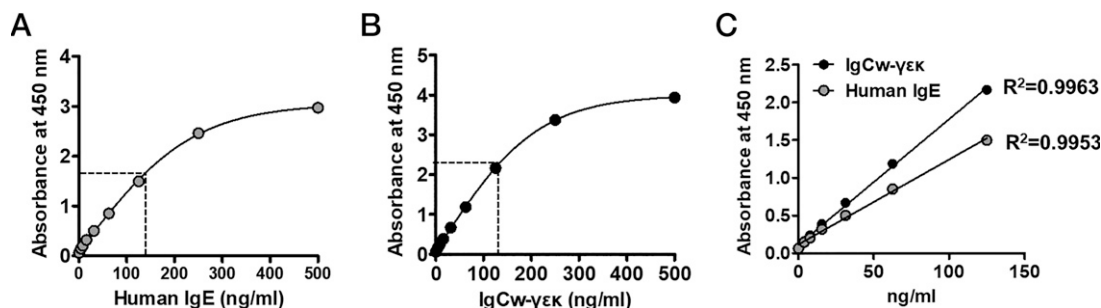
FIGURE 5. Standard curves generated using the respective polyclonal human IgE and IgCw-γεκ molecules. Standard curves were generated using known concentrations of polyclonal human IgE (**A**) and IgCw-γεκ (**B**). Polyclonal human IgE and IgCw-γεκ were placed in wells coated with a capture Ab. Bound Ig molecules were detected using an IgE Human Uncoated ELISA Kit as described in *Materials and Methods*. (A and B) Logistic regression curves. The linear range is denoted by the dotted line. (**C**) Linear equations from (A) and (B). Data are presented as the mean ± SD of three independent experiments.

Table II.   IgE concentration measured using two standard curves

| | | Concentration (ng/ml) Determined by Interpolating y Values on the Standard Curve IgCw-γεκ | |
Samples of Unknown Concentration	Human IgE	Not Normalized	Normalized (× 1.462)[a]
6C407 IgE	70.42	51.55	75.366
3D8 IgE	25.01	16.92	23.737
Human plasma	344.93	237.29	346.951

[a]Normalized by multiplying the original concentration by the ratio of the molecular masses (1.462:1 = ratio of IgE [~190 kDa] to IgCw-γεκ [~130 kDa]).

IgE. IgCw-γεκ also was able to inhibit in vitro sensitization of RBL-2H3-hFcεRIα cells by IgE in a dose-dependent manner (Fig. 4D–F). Coincubation of IgCw-γεκ with an equivalent concentration of IgE inhibited sensitization of RBL-2H3 cells, along with subsequent β-hexosaminidase release upon cross-linking of IgE, by 72% (Fig. 4D). Similarly, when IgCw-γεκ was added to cells preincubated with an equivalent concentration of IgE, the former inhibited β-hexosaminidase release by 31% (Fig. 4E). It is surprising that IgCw-γεκ inhibited degranulation of cells already sensitized by IgE. So far, all studies examining the inhibitory effect of competitors on IgE sensitization produced positive results only when cells were pretreated with inhibitors or coincubated with a competitor and IgE. To our knowledge, no study has reported the results obtained by preincubating cells with IgE prior to exposure to a competitor.

Commonly, in vitro inhibition of IgE-mediated sensitization is determined by measuring inhibition of histamine or β-hexosaminidase release from cultured basophils (or mast cells). In vivo, inhibition is determined by measuring inhibition of the Prausnitz–Küstner (P-K) reaction, a form of passive cutaneous anaphylaxis. To date, numerous recombinant human Fcε fragments produced in *Escherichia coli* and mammalian cells (22–25) require high molar concentrations to inhibit sensitization by IgE in vitro and in vivo. Studies show that an *E. coli*–derived Fcε2–4 fragment was 4-fold less effective than IgE at inhibiting histamine release in vitro (22), and that a 200-fold molar excess of the Fcε2–4 fragment over that of IgE was required to inhibit the P-K reaction (23). An *E.coli*–derived Fcε301–376 fragment had to be used at a concentration 11–13-fold higher than that of IgE to inhibit histamine release by 50% in vitro, and a 10-fold higher molar concentration was required to inhibit the P-K reaction by 50% (24). Fcε315–547 and Fcε329–547 fragments expressed in mammalian cells were 2–4-fold less effective than IgE to inhibit histamine release in vitro (25). It is worth noting that the potency of IgCw-γεκ was only slightly less, or almost equivalent to (on a molar basis), that of IgE with respect to inhibiting in vitro sensitization by IgE (Fig. 4). Further in vivo studies are needed to prove that IgCw-γεκ is clinically useful as a potent antagonist of IgE binding to mast cells.

Human IgE is required for in vitro assays that measure allergic responses and IgE concentrations. The range of total sIgE (tIgE) among nonatopic and atopic individuals overlaps; indeed, elevated tIgE does not directly correlate with allergic manifestations. Thus, allergen-specific sIgE is used primarily as an indicator of allergy (26). Even if quantification of tIgE alone has limited value as an indicator of allergy, measurement of tIgE measurement is useful for establishing effective dosing of allergic patients receiving omalizumab (humanized IgG1-specific human IgE-Fc) therapy (8) and for determining IgE-sp. act. (expressed as the ratio of sIgE to tIgE), which is helpful for translating IgE responses into allergic symptoms (26). A total IgE calibration curve is also used to measure sIgE levels; the measured concentration is interpolated from a tIgE reference curve (this is because there are no internationally accepted sIgE references). To minimize differences in tIgE results obtained from different laboratories, assays for human serum tIgE are all calibrated against the third World Health Organization IgE international standard. The most recent is the third International Reference Preparation (IRP), coded 11/234 (https://www.who.int/biologicals/BS_2220_Candidate_Preparation.pdf) (27). Lack of stocks of the second IRP led to the preparation of a third IRP stock from pooled sera (and plasma) from individuals; this is dispensed and lyophilized in ampules with an assigned value of 13,500 IU/ml (0.0324 mg/ml) (27). Although this product has been tested and found to be negative for hepatitis B surface Ag, anti-HIV, and hepatitis C virus RNA, it should be regarded as a potentially hazardous biological agent and handled safely in the laboratory. By contrast, IgCw-γεκ is purified from HEK293F cells cultured in serum-free medium; therefore, it need not be considered as a biological agent. This advantage might make IgCw-γεκ a promising alternative to the World Health Organization IgE International standard for assays that measure IgE concentrations.

Disclosures

The authors have no financial conflicts of interest.

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