Original Articles

Isolation and Functional Characterization of Recombinant GAD65 Autoantibodies Derived by IgG Repertoire Cloning From Patients With Type 1 Diabetes

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The generation of human monoclonal autoantibodies is critical for understanding humoral immune responses in autoimmunity. In this study, we isolated the first human recombinant antibodies to glutamate decarboxylase (rGAD65ab) by IgG repertoire cloning, phage display of Fab fragments, and biopanning from two patients at onset of type 1 diabetes. We demonstrate that natural Ig heavy- and light-chain pairings of autoantibodies can be isolated by the recombinant approach and have a major selection advantage over other rGAD65ab. Among eight rGAD65ab, three (rGAD65ab A–C) displayed all functional and structural properties of known disease-related GAD65ab, including reactivity in the enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), islet cell antibody (ICA) test, and variable gene usage. Dominant epitope recognition was directed to the previously defined epitope EP-1 in the middle of GAD65, corroborating its immunodominance in the molecule. New features, such as assay-dependent GAD65 reactivity and new epitope recognition, were observed in two rGAD65ab (D and E). These antibodies were positive in the GAD65 ELISA and ICA test but not in the GAD65 RIA, providing the first examples for ICA with incongruent results in solid-phase and fluid-phase assays. In conclusion, phage display–derived antibodies reflected well the natural autoantibody response in type 1 diabetes and may allow further characterization of assay-dependent features of GAD65ab and the recognition of epitopes in solid- but not fluid-phase assays. ***Diabetes*** **50:1976–1982, 2001**

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BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish-peroxidase; IA2, islet antigen 2; ICA, islet cell antibody; MICA, monoclonal islet cell antibody; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PBS-T, PBS–0.5% Tween PCR, polymerase chain reaction; Phab, recombinant phage; PMSF, phenylmethylsulfonyl fluoride; rFab, recombinant Fab fragments; rGAD65ab, recombinant antibody to GAD65; RIA, radioimmunoassay.

Autoantibodies against cytoplasmic islet cell antigens are a hallmark of type 1 diabetes and represent well-established predictive and diagnostic markers for the development of the disease (1–4). Islet cell antibody (ICA) reactivities are directed to two major autoantigens, GAD65 (5) and the protein-tyrosine-phosphatase–like protein islet antigen 2 (IA2) (6,7). Ongoing efforts are made to define the epitopes and variable gene usage of such autoantibodies (8–14) and the role of autoreactive B cells in the pathogenesis of diabetes (15,16). Repertoire cloning methods and phage display have been used successfully to produce human recombinant autoantibodies from patients with diverse autoimmune diseases (17–24). The functional properties of these artificial antibodies may mirror the in vivo immune response to a certain extent. Because combinatorial antibody libraries randomly recombine heavy and light chains, the issue is whether this approach leads to the recovery of original pairs of H and L chains and, thus, to disease-relevant autoantibodies (25–28). By generating new diabetes-related recombinant antibodies against GAD65 and IA2 via construction and selection of an IgG1-Fab phage display library, we wanted to complement the repertoire of diabetes-related autoantibodies and address the issue of whether recombinant antibodies obtained by repertoire cloning and antigen-specific biopanning display functional and structural characteristics similar to naturally occurring disease-related antibodies and can thereby help to define the autoantibody response in type 1 diabetes.

RESEARCH DESIGN AND METHODS

Patient samples. Peripheral blood and sera from two women (age 33 and 34 years, respectively) whose diabetes was newly diagnosed were obtained after informed consent of the donors. Titers of ICA, GAD65 autoantibodies (GAD65ab), and IA2c-specific autoantibodies (IA2c-ab) were determined by immunohistochemistry, radioimmunoassay (RIA), or enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells (PBMC), used as a source of mRNA for the generation of an IgG1-Fab library (29), were isolated by density gradient centrifugation.

Polymerase chain reaction amplification and library construction. Total RNA was extracted from 1×10^8 PBMC by standard guanidinium isothiocyanate extraction (30). Reverse transcription of RNA (10 μg) to single-stranded cDNA using 400 ng oligo$(dT)_{16-18}$ primer (Amersham Pharmacia Biotech) and independent amplification of cDNA of IgGγ1 heavy chains, κ-chains, and

λ-chains with a primer set purchased from Stratacyte was performed by polymerase chain reaction (PCR) as described previously (18,29). For the heavy-chain Fd region, six upstream primers for the six VH families (codons −2 to 8, numeration according to Kabat et al. [31]), were used in six independent reactions. One λ-chain and one κ-chain upstream primer (λ-chains: codons −7 to 5; κ-chains: codons −4 to 8) were used to amplify separately the light chains. The downstream primers were designed to match with the constant domain sequences (codons 226–223 of CH1, codons 210–214 of Cκ or Cλ region). PCR mixes of 100 μl contained 5–10 μl of first-strand cDNA and 60 pmol of the respective primers. The mixes were subjected to a hot-start PCR (42 cycles with a 9600 thermocycler [Perkin Elmer]: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, final extension at 72°C for 10 min) using Ampli-*Taq* polymerase (Perkin Elmer). Equal amounts of purified heavy- and light-chain PCR products were pooled and cut with *Xho*I/*Spe*I and *Sac*I/*Xba*I, respectively (Roche) for library construction as described previously (29). Phagemid vector pComb3HSS was provided by Dr. Carlos Barbas III. The light-chain DNA was cloned into pComb3HSS and transformed into *Escherichia coli* XL1-Blue cells (Stratacyte) by electroporation. The DNA of Fd fragments then was inserted into the light-chain library, and library phages were produced and concentrated by precipitation (29). For each transformation, the number of colony-forming units was determined, and the proportion of recombinant clones was assessed by double digestion of miniprep DNA with *Xho*I/*Spe*I and *Sac*I/*Xba*I, respectively.

Phage cloning of monoclonal ICAs 2 and 4. The DNA encoding light chains and Fd regions of two monoclonal GAD65-specific B-cell lines, monoclonal ICA 2 (MICA2) and MICA4 were cloned separately into pComb3HSS as described for the library. For the amplification of the MICA4 heavy chain, a *Sal*I introducing forward 5′primer (VHBsal: 5′AGGTCGACCTGCAGGAGTC GGG3′) was used with CH1 to avoid restriction of the variable-region DNA of MICA4 carrying an *Xho*I-site. The combination of correct heavy and light chains (MICA2 H + L, MICA4 H + L) and the combination MICA4 H/MICA2 L were cloned. The recombinant Fab-displaying library phages (Phab) were designated rMICA2 and rMICA4 and rMICA4 H/2 L, respectively.

Selection of the combinatorial library on GAD65 and IA2c. After precipitation, Phab were resuspended in 4 ml of phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2 mmol/l KCl, 6 mmol/l Na_2HPO_4, and 1.4 mmol/l KH_2PO_4 [pH 7.2]) with 1% bovine serum albumin (BSA; Sigma) and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF; Sigma). Five wells of a Maxisorb microtiter plate (Nunc) were coated with 1.25 μg/well affinity-purified GAD65 from baculovirus expression (32) in PBS for 1 h at 37°C. The selection and amplification of phages were done essentially as described previously (29), and PBS–0.5% Tween (PBS-T) was used for washing. Approximately $1 \times 10^{10-11}$ Phab were added to the antigen-coated wells (blocked with 3% BSA in PBS-T) and incubated at 37°C for 1.5 h. Unbound phage were removed by vigorous washing with an excess of PBS-T (4°C) three times in the first round. Washing stringency was increased to 5 and 10 washing steps in the second and third rounds, respectively.

Selection of Phab on human biotinylated IA2c (IA2cbio) was performed by solid-phase panning with streptavidin-coupled plates (Pierce) coated with 1.25 μg/well biotinylated IA2c in TBS–0.05% Tween–0.1% BSA (TBS: 10 mmol/l Tris/HCl [pH 7.4], and 150 mmol/l NaCl) for 1 h at 37°C. In a second approach, Phab were selected for binding to IA2cbio in solution essentially as described by Griffiths et al. (33).

Epitope-directed panning. The unselected library was panned on GAD65 in which one epitope was blocked by purified MICA4. Because GAD65 tends to form dimers, MICA4 was used as capture ab for GAD65 and as competing ab in solution. All incubation steps were done for 1 h at 37°C. Four microtiter wells were coated with 3.75 μg/well MICA4 in PBS. After the wells were blocked with 3% BSA in PBS-T, the wells were incubated with 1.25 μg/well GAD65, washed twice with PBS-T, and again incubated with an excess of MICA4 (3.75 μg/well). In the presence of MICA4, $1 \times 10^{10-11}$ Phab preblocked with MICA4 Ig were applied to the epitope-masked sandwich. Five selection rounds with 10 washing steps were performed.

Expression of soluble Fab. Selected phage clones were reengineered for expression of soluble Fab as described previously (29). In short, isolated phagemid DNA preparations were subjected to *Spe*I/*Nhe*I restriction to remove the geneIII sequence. After purification, the DNA (~4,150 bp) was self-ligated and electroporated into XL1-Blue. rFab were expressed from single colonies and induced with 1 mmol/l IPTG (isopropyl-thio-β-d-galactosid) (BioMol). After incubation at 30°C for 16 h, bacteria were pelleted and resuspended in 1 ml of lysis buffer (50 mmol/l Tris-HCl [pH 8.0], 150 mmol/l NaCl, 5 mmol/l $MgCl_2$, 1 mmol/l PMSF, 10 mg/ml lysozyme, and 25 units DNase I [Roche]). After 30 min of incubation at 37°C, the bacteria were sonicated (30 × 1-s bursts at 50% power), and cellular debris was removed by centrifugation. The supernatants of these lysates were analyzed for Fab production and reactivity in all binding assays.

ELISA procedures. All conjugates were preblocked for 30 min at 37°C with XL1-Blue lysates or XL1-Blue lysates mixed 1:1 with a solution of $\sim 1 \times 10^{12}$ VCS-M13 immobilized onto nitrocellulose-membranes to minimize cross-reactivity (34). For monitoring rFab expression, microtiter plates were coated with 100 ng/well goat anti-human $F(ab')_2$ specific antibody (Dianova) in PBS. All incubation steps were performed for 1 h at 37°C, and PBS-T was used for washing. Wells were washed once and blocked with 3% BSA in PBS-T. Soluble Fab containing supernatants and a Fab standard (Dianova) were diluted and incubated on the capture antibody. After washing eight times, bound rFab were detected with horseradish-peroxidase (HRP)-labeled goat anti-hum-$F(ab')_2$ conjugate (1:1,000). Then, wells were washed eight times, and substrate was added (100 mmol/l Na-Acetate [pH 4.2], 50 mmol/l $NaH_2PO_4 \times 1H_2O$ containing 0.11 g/l ABTS [2,2′-Azino-di-(3-ethylbenzthiazoline-sulfuric acid-6] 2.5 mmol/l H_2O_2, and 1 mg/ml substrate enhancer (Roche). Specificity ELISAs were performed as described above using 100 μl of the following pure antigens diluted to 2 or 10 μg/ml in PBS (pH 7.4) for coating: human GAD65 and human GAD67 from baculovirus expression, human insulin (Sigma), BSA (Sigma), and MICA4 IgG. Human IA2cbio was coated in TBS–0.05% Tween–0.1% BSA to streptavidin-coupled microtiter plates and blocked with 20 mmol/l Tris/HCl, 300 mmol/l NaCl, 2 mmol/l benzamidine (Sigma), 2 mmol/l EDTA (Sigma), 1 mmol/l PMSF, 1% BSA, and 0.1% Tween.

RIA. In vitro transcription and translation of antigens was performed using the TNT Sp6 or TNT T7 coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S] methionine (40 μCi, 1,000 Ci/mmol [Amersham Pharmacia Biotech]) as described previously (4). The full-length cDNAs of human GAD65 in pcDNAII (provided by Dr. A. Lernmark), human GAD67 in pSV.Sport1 (10), the cDNA encoding the cytoplasmic part of IA2 (amino acid 603–979) in pSP64polyA (provided by Dr. M.C. Christie), and the cDNA of four different chimeric GAD65/GAD67 molecules (10) were used. Briefly, 5 μl of rFab were bridged with 5 μl of goat anti-human $F(ab')_2$ antibody (Dianova) and incubated in duplicates for 16 h at 4°C with the radioactive antigens. Supernatants (2.5 μl) containing MICA IgG, polyclonal anti-GAD67ab K2 from rabbit (provided by DPC Biermann, Berlin), anti-GAD65/GAD67 monoclonal antibody 3,243 from mouse (provided by Dr. B. Ziegler), or 2.5 μl of a IA2c-reactive human standard serum were used as positive controls. Normal human serum, unrelated rFab, and preparations of pComb3HSS were applied as negative controls. Protein A–Sepharose (1 μg) was added. After 1.5 h at 4°C, unbound proteins were removed by washing 15 times in membrane-bottomed microtiter wells (0.6-μm pore size; Millipore) before measuring counts per minute of captured immune complexes in a beta counter.

Immunohistochemistry. Unfixed cryostat sections (5 μm) of human pancreas were incubated with 35 μl of rFab or MICA culture supernatant for 1 h at 22°C. Sections were washed three times for 5 min with PBS (pH 7.4), then incubated with a biotin-labeled anti-human-$F(ab)_2$-conjugate (Sigma) diluted 1:500 in PBS for 1 h. After washing as described above, Cy_2-labeled streptavidin (Dianova) diluted 1:500 in PBS was applied to the sections for 30 min. Staining was visualized after washing and mounting in a 3:1 mixture of PBS/glycerol (vol/vol) with a Zeiss immunofluorescence microscope .

Blocking studies. For epitope characterization (14) of rFab, cryostat sections of human pancreas or rat cerebellum were preincubated for 1 h at 22°C with 50 μl of rFab, unrelated rFab, and human Fab standard (Dianova) diluted in XL1-Blue lysate. After two washing steps for 5 min with prechilled PBS, the pretreated sections were incubated with 30 μl of an appropriately diluted MICA antibody for 1 h and washed again as described above. Binding of the MICA IgG was detected with an Fc-specific biotin-labeled anti-human–IgG conjugate (Dianova) diluted 1:200 in PBS followed by washes and incubation with Cy_2-labeled streptavidin as described above.

Sequencing of heavy- and light-chain variable regions. Sequencing of both DNA strands was carried out according to the dideoxy chain termination method on a 373A DNA Sequencer (Applied Biosystems) using an FS-*Taq* fluorescent dideoxynucleotide terminator sequencing kit (Applied Biosystems). Vector-specific 5′ sequencing primers were C3HSS2720s: 5′ ATTGTTATTAC-TCGCTGC 3′ (VH) and pC3HSS2575s: 5′ TGAGCGGATAACAATTGA 3′ (VL). The 3′ sequencing oligonucleotides were CH73as 5′ TCCTTGACCAGGCAGC-CCAG 3′(VH), CLκas: 5′ GATGAAGACAGATGGTGCAG 3′ (Vκ) and CLλas: 5′ GGGAACAGAGTGACCGAGGG 3′ (Vλ). Identity searches of the variable gene segments were performed using the VBASE directory (35).

RESULTS

Construction of a diabetes-related IgG phage display library. IgG repertoire cloning was performed based on peripheral B cells pooled from two individuals at onset of type 1 diabetes. Both patients showed high serum titers of ICA (>640 Juvenile Diabetes Foundation units), were high-

ly positive for GAD65ab, and showed medium reactivity with IA2 in fluid-phase (RIA) and solid-phase (ELISA) assays. Because well-characterized GAD65-reactive B-cell clones (MICA1–6, MICA8–10) had been obtained previously from the same blood donation of both participants at onset of the disease (10,36), a high frequency of diabetes-related autoreactive B cells was expected in their circulation.

The size of the light-chain library generated from RNA extracted from 10^8 PBMC comprised 5×10^6 transformants with an insert rate of 100%. Although equal amounts of λ- and κ-chain PCR products had been used for ligation, 74% of the recombinant clones contained a λ light chain, as assessed by PCR ($n = 31$ clones). Cloning of pooled Ig VH fragments of six different VH families into this light-chain library yielded the final combinatorial library consisting of $\sim 2 \times 10^6$ independent clones, with 93% of them carrying heavy- and light-chain inserts.

Correct heavy- and light-chain combination of MICA2 and MICA4 restores GAD65 reactivity. To verify GAD65 reactivity of naturally occurring GAD65ab on phage and to obtain controls for detection of GAD65-reactive recombinant Fab fragments, we cloned MICA2 and MICA4 heavy- and light-chain genes into pComb3HSS in their correct (2 H/2 L, 4 H/4 L) and crossed-over combination (4 H/2 L). MICA2 and MICA4 were chosen because the former recognized with medium functional affinity (avidity 1.4×10^8 Mol^{-1}) a Western blot–positive GAD65 epitope and contained a λ light chain, whereas the latter bound with high avidity (2.6×10^9 M^{-1}) a conformational epitope in GAD65 and represented a κ light chain (8,32). The heavy-chain variable region of MICA4 was destroyed by the conventional cloning procedure but could be rescued by switching to an alternative restriction enzyme. This demonstrates that some naturally occurring antibodies may be destroyed during library construction. Consequently, the MICA4 heavy chain cannot be expected as a component of the combinatorial library from the patients. Only the natural light- and heavy-chain combinations revealed GAD65 reactivity, whereas the rMICA4 H/2 L phage and Fab were negative (Fig. 1). Similar loss of antigen reactivity after substitution of light chains is known for thyroid peroxidase–specific Fab from immune libraries (17), for murine antibodies against a glycopeptide antigen (37), and for the restrictive pairing of anti-DNA antibodies in systemic lupus erythematosus (21).

Naturally occurring autoantibodies can be isolated by phage display. During GAD65-specific biopanning in ELISA wells, enrichment of antigen-specific Phab in the diabetes-related phage display library steadily increased, as monitored by phage recovery from rounds 2–5 (data not shown). Although none of the 93 individual Phab eluted from the third round of panning was positive in the GAD65 ELISA, the number of GAD65-binding Phab increased to 54% and finally to 98% after rounds 4 and 5, respectively. Sequencing of all GAD65-reactive clones revealed complete sequence identity in V, D, and J regions of heavy and light chains in all clones, indicating that they were derived from one initial GAD65-specific phage preferentially enriched during panning. This clone was designated rGAD65ab A. Sequence comparison to MICA heavy- and light-chain genes demonstrated that the heavy-chain variable region of rGAD65ab A was identical to that of MICA6. The

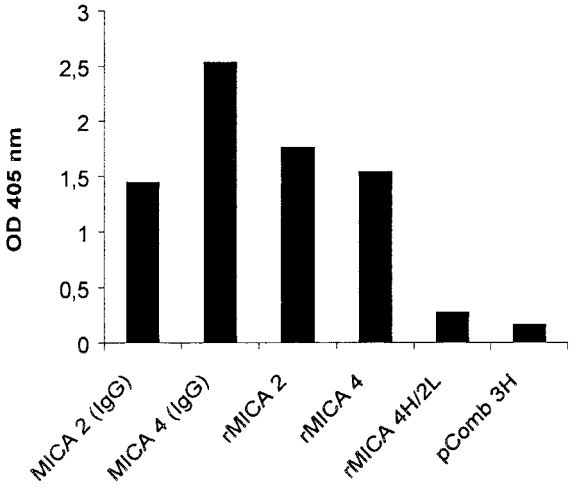

FIG. 1. Correct heavy- and light-chain combination of MICA2 and MICA4 restores GAD65 reactivity. GAD65 reactivity of antibodies was analyzed by ELISA on plates coated with 5 μg/ml GAD65 and bound antibodies detected by HRP-labeled anti-human-F(ab)$_2$ specific antibody. MICA2 IgG (25 ng) and MICA4 IgG (10 ng) from culture supernatant of the Epstein-Barr virus–transformed B-cell lines were used as positive controls. Recombinantly expressed rMICA2 (2 H/2 L), rMICA4 (4 H/4 L), but not the crossed-over heavy- and light-chain combination MICA4 H/MICA2 L recognized GAD65 as soluble rFab derived from bacterial lysates. The pComb3H vector served as a negative control.

light chain was almost identical to the MICA6 λ chain and missed only four of the seven amino acid exchanges that had accumulated during affinity maturation in the MICA6 light chain compared with the germline gene. This suggests that this light chain was derived from a precursor cell of the MICA 6 clone and demonstrates that a disease-related natural human autoantibody had a major selection advantage in the phage display approach.

New GAD65-reactive Phab obtained by epitope-specific biopanning. The abundance of rGAD65ab A may be explained by two alternatives: this phage was the only GAD65-reactive recombinant clone contained in the library, and/or because of its affinity or epitope recognition, rGAD65ab A phages may have competed out other GAD65-reactive clones during panning. To test both alternatives, we designed and performed epitope-specific panning of phages in the presence of an antibody that blocked the MICA6 epitope. MICA4 was selected for blocking because it prevented binding of MICA6 to GAD65 in tissue sections of human pancreatic islets (14,36) and had a higher avidity for GAD65 than MICA6 (32).

The primary unselected combinatorial library then was panned on the MICA4/GAD65/MICA4 sandwich in the presence of soluble MICA4. Sequence analysis of 21 obtained positive clones revealed again the rGAD65ab A sequence in 13 samples. Seven different recombinant Ig gene combinations designated rGAD65ab B–H were identified in the remaining samples, which represented new combinations of heavy and light chains not found in the MICA. In conclusion, the natural heavy- and light-chain combination in rGAD65ab A had a strong selection advantage over other GAD65-reactive clones contained in the library.

No enrichment of IA2-reactive phages. Selection of the original amplified library on solid-phase purified IA2c by the same methods or with biotinylated IA2c coupled to streptavidin-coated magnetic beads did not yield any IA2-

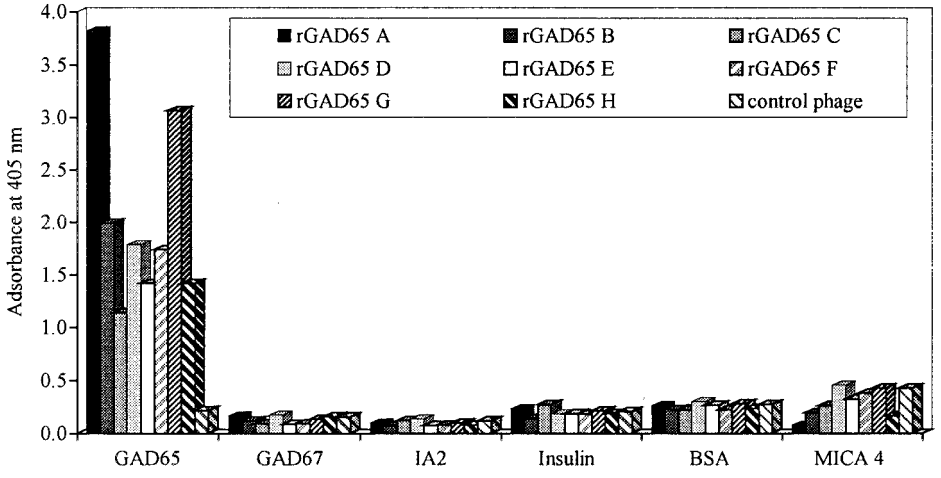

FIG. 2. The rGAD65ab A–H phages are specific for GAD65. Cross-reactivity of rGAD65ab A–H phages with a panel of solid-phase antigens was analyzed by ELISA. Phages were incubated in duplicate in microtiter wells coated with 50 μl of antigen (10 μg/ml). Binding of Phab to all antigens except the competing antibody MICA4 was detected with an HRP-labeled anti-human $F(ab')_2$ antibody diluted 1:1,000. Binding of Phab to MICA4 was monitored using anti-VCSM 13 antibody (1:10,000) and anti-rabbit IgG HRP-labeled antibody (1:50,000). The control phage is a representative negative clone.

reactive clones. This is consistent with the finding that no IA2-reactive natural human B-cell clones could be isolated by conventional methods from both patients, despite extensive screening (W. Richter, unpublished observation). In conclusion, the frequency of circulating IA2-specific B cells is low in the selected patients, despite medium serum titers of IA2 antibodies. Obviously, combination of heavy and light chains not involved in IA2 binding in vivo did not yield IA2-reactive antibodies on phage fortuitously.

rGAD65ab A–H Phab are GAD65-specific. Cross-reactivity of rGAD65ab A–H with the blocking agent BSA, with other human islet cell antigens, such as insulin and IA2c, or with the larger GAD isoform GAD67 was investigated further by ELISA (Fig. 2). All Phab specifically recognized only GAD65 and, thus, showed a reactivity typical for naturally occurring monoclonal GAD65ab associated with type 1 diabetes, which do not all cross-react with GAD67 (8,38).

GAD65 reactivity of some rGAD65ab Fab fragments is assay-dependent. All naturally occurring MICAs bind GAD65 on solid phase (ELISA), in fluid phase (RIA, immunoprecipitation), and in their natural localization on tissue sections. To compare the functional performance of the recombinant antibodies rGAD65ab A–H with that of the MICAs, we reconstructed the phagemid DNA for production of soluble Fab fragments in *E. coli*, which then were tested for their GAD65 reactivity in different assay formats. It is surprising that only rGAD65ab A–E retained their GAD65 reactivity when expressed as soluble Fab, whereas rGAD65ab F–H lost it (Table 1). We can only speculate that part of the phage surface may have been required to mediate GAD65 binding in clones F–H or that these rFab fragments did not fold correctly into intact molecules when expressed without the fusion partner gene III. When GAD65ab A–H and rMICA2 were tested in the classical ICA test on cryostat sections of human pancreas (Table 1), the rGAD65ab A–E and the rMICA2 Fab revealed a staining pattern like the MICA. In a RIA based on immunoprecipitation of radiolabeled GAD65 by rFab coupled to PAS via an anti-human $F(ab')_2$ fragment–specific antibody, rGAD65ab A–C and rMICA2 were positive (Table 1). In sum, only three of the eight rGAD65ab (rGAD65ab A–C) showed GAD65 reactivity in all tests similar to that of known GAD65 autoantibodies, whereas the GAD65 reactivity of rGAD65ab D and E Fab was assay-dependent.

rGAD65ab A–E recognize common and new epitopes of GAD65. Assay-dependent differences in GAD65 recognition may be explained by the epitope recognition of the soluble Fab fragments. Thus, histochemical epitope analysis, was performed on human pancreas sections that were blocked with the distinct rFab A–E before the access of MICA1–10 to their GAD65 epitope was assessed. rGAD65ab A–C blocked the binding of MICA4, MICA6, and MICA10 to epitope cluster EP-1, located in the middle of

TABLE 1
Binding characteristics and epitope reactivity of GAD65-specific Phab and Fab in different assays

Clone	Phab on phage	Soluble Fab				
	ELISA	ELISA	Histochemical test	RIA	Blocking of indicated MICA	Reactivity with GAD65/67 chimera*
rGAD65ab A	+	+	+	+	4/6/10	C1/C4
rGAD65ab B	+	+	+	+	4/6/10	C1/C4
rGAD65ab C	+	+	+	+	4/6/10	C1/C4
rGAD65ab D	+	+	+	−	None	NP
rGAD65ab E	+	+	+	−	None	NP
rGAD65ab F	+	−	−	−	NP	NP
rGAD65ab G	+	−	−	−	NP	NP
rGAD65ab H	+	−	−	−	NP	NP
rMICA2	+	+	+	+	2/7	C1/C3

*Chimeric GAD65/67 molecules: C1, $GAD67_{1–252}/GAD65_{245–585}$; C3, $GAD67_{1–457}/GAD65_{450–585}$; C4, $GAD65_{1–499}/GAD67_{458–594}$; NP, not possible because unreactive in histochemistry or RIA.

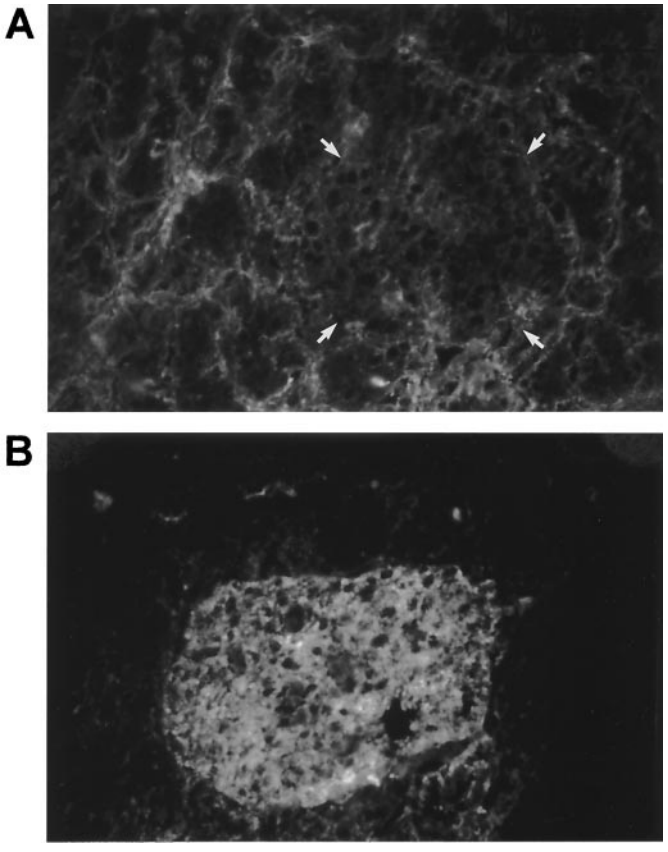

FIG. 3. Epitope recognition of rFab from rGAD65ab A and D. Binding of the rGAD65ab A and D to GAD65 in its natural localization in islet cells was analyzed in an epitope-specific histochemical blocking test. Human pancreas sections were blocked with the distinct rFab before the access of MICA1–10 to their GAD65 epitope was assessed by a biotinylated Fc-specific antibody and streptavidin-Cy^2 conjugate. rGAD65ab A blocked the binding of MICA4, MICA6, and MICA10 to the islets (A = MICA4), whereas rGAD65ab D did not inhibit the binding of any of the MICAs (B = MICA4).

GAD65 (10,14), whereas rGAD65ab D and E did not inhibit the binding of any of the MICAs (Fig. 3, Table 2). Control antibody rMICA2 blocked binding of MICA2 and MICA7 and recognized epitope cluster EP-3 as expected (10,14). For the RIA-positive antibodies rGAD65ab A–C and rMICA2, epitope recognition was confirmed by immunoprecipitation of chimeric GAD65/GAD67 molecules (Table 1). In conclusion, epitope-specific panning in the presence of EP-1 blocking antibodies still allowed part of the new rGAD65ab to be enriched on the MICA4/MICA6 epitope cluster (EP-1). This suggests that EP-1 is an immunodominant epitope in GAD65, a notion that is strongly supported by the reactivity of sera of GAD65ab-positive patients, which identified EP-1 as the most frequently detected GAD65 epitope (14). The assay-dependent binding of rGAD65ab D and E thus correlated with the binding of new epitopes not covered by MICA1–10.

Ig Immunoglobulin variable gene usage of rGAD65ab A–E. The panel of natural human GAD65 autoantibodies with known nucleotide sequences comprises more than 10 antibodies generated by conventional techniques (32,38). In comparing the variable gene usage and mutation rates of heavy and light chains of recombinant and natural GAD65 antibodies, many similarities were evident (Table 2) (32,38). Besides the near identical sequences of rGAD65ab A and MICA6, rGAD65ab C used the same heavy-chain like MICA2 (Table 2), which, however, had accumulated nine additional replacement mutations not found in rGAD65ab C. This suggests that the rGAD65ab C heavy chain is derived from a precursor cell related to the MICA2 clone, which represents earlier stages of affinity-driven B-cell selection. Because the λ chain in rGAD65ab C was different from that in MICA2, this rFab most likely represents an example for heavy-chain promiscuity, i.e., the ability of a heavy chain to form a functional antibody fragment by pairing with different light chains. It is surprising that the same light chain was also used in rGAD65ab E in combination with a VH4 DP71 gene arguing in favor of light-chain promiscuity. In sum, the V gene usage demonstrated that three of five recombinant GAD65ab (A, C, and E) and four of nine MICA (1, 2, 6, and 9) combined a VH4 heavy chain with a λ light chain, suggesting that a VH4/λ chain gene usage is dominant in the repertoire of GAD65-specific B cells of these two patients.

DISCUSSION

By IgG repertoire cloning of B cells from two individuals with autoimmune diabetes, phage display, and biopanning on the major diabetes autoantigens GAD65 and IA2c, we obtained new recombinant GAD65 antibodies that reflected well the circulating B-cell population in the patients. Whereas GAD65-specific antibodies were ferquently detected, IA2c-reactive antibodies could not be obtained. Thus, parallel to lower IA2-ab serum levels, IA2-reactive B cells circulated at a low frequency in the peripheral blood of these two patients with type 1 diabetes.

Among the eight recombinant GAD65-reactive phages obtained in this study, three reflected all typical features of naturally occurring GAD65 autoantibodies (rGAD65ab A–C). Two antibodies displayed some new features not seen with the MICA (rGAD65ab D and E), and three phages have to be considered as artificial because they lost GAD65 reactivity when expressed as rFab. The most dominant clone, rGAD65ab A, was disease-related and contained the original heavy- and light-chain pairing of a MICA6 precursor cell. Thus, we demonstrated for the first time that a natural heavy- and light-chain combination has a major selection advantage over all other antigen-reactive phages

TABLE 2
V gene usage of GAD65-specific rGAD65ab A–E and MICA1–10

Clone	Heavy-chain V-, D-, J-region genes	Light-chain V-, J-region genes
rGAD65ab A	VH 4, DP 79/DLR2/JH6b	Vλ 2/hslv2046/Jλ 2/3
rGAD65ab B	VH 1, DP 88/—/JH5b	Vλ 2/DPL 11/Jλ 3b
rGAD65ab C	VH 4, DP 71/DLR 2/JH4b	Vλ 3/V2-17/Jλ 3b
rGAD65ab D	VH 2, DP 26/DN 4/JH5b	Vλ 2/DPL 11/Jλ 2,3
rGAD65ab E	VH 4, DP 71/DN 4/JH3b	Vλ 3/V 2-17/Jλ 3b
MICA1	VH 4, DP 71/DK 1/JH2	Vλ 2/DPL 11/Jλ 2,3a
MICA2	VH 4, DP 71/DLR 2/JH4b	Vλ 1/DPL 2/Jλ 2,3a
MICA3	VH 1, DP 7/DK 4/JH5b	Vλ 2/DPL 13/Jλ 1
MICA4	VH 5, DP 73/DN1/JH5b	Vκ 3/L 16/Jκ 4
MICA5	VH 1, DP 14/—/JH4b	Vκ 2/DPK18/Jκ 1
MICA6	VH 4, DP 79/DLR2/JH6b	Vλ 2/hslv 2046/J λ 2,3a
MICA7	VH 1, DP 10/DXP′1/JH4b	Vκ 1/L 12a/Jκ 1
MICA9	VH 4, DP 79/D 6–25/JH5b	Vλ 1/DPL 2/J λ 2/3
MICA10	VH 3, DP 38/DXP4/JH3a	Vλ 1/DPL 8/J λ 2/3

during solid-phase panning. This makes IgG repertoire cloning and isolation of phage display–derived antibodies an attractive approach for further characterization of the diabetes-related autoantibody response.

Like the recombinant control antibody rMICA2, rGAD65ab A retained its GAD65 reactivity in ELISA, RIA, and the ICA test and bound to the same epitope like its natural counterpart. rGADab B and C, which displayed identical features and epitope recognition like rGAD65ab A, used unrelated variable gene regions that, for rGAD65ab C, were almost identical to the MICA2 heavy chain. Again, only a few replacement mutations that had accumulated in the natural counterpart were missing in the rGAD65ab C heavy chain. In combination with a new light chain, however, this antibody now bound to the dominant middle epitope in GAD65 (EP-1), which is distinct from that bound by MICA2. This, surprisingly, demonstrates that promiscuity in the usage of heavy or light chains can retain reactivity of an antibody to the same antigen but may alter its fine specificity in the target molecule. The EP-1 cluster of epitopes, to which we found dominant enrichment of recombinant GAD65ab, was also a dominant target of GAD65 antibodies in the patients' sera (14).

With rGAD65ab D and E, we observed an assay-dependent GAD65 reactivity parallel to a new epitope recognition not covered by MICAs. Both antibodies were positive for GAD65 in the solid-phase assays (ELISA and histochemistry) but negative with soluble GAD65 in the RIA. Because the same secondary antibody was used for ELISA detection and for coupling of the rFab to PAS for all rGAD65ab, technical problems of Fab recognition or immune complex binding cannot explain these results. Either the epitopes recognized by rGAD65 D and E are accessible only on solid-phase GAD65, or the new epitopes are recognized less efficiently in liquid phase as a result of a lower affinity of these antibodies for GAD65. Strong selection for phages binding to GAD65-coated ELISA wells during the repeated panning procedures may have favored the isolation of antibodies such as D and E. Natural antibodies with similar reactivity may occur in human sera and could explain why solid-phase assays, such as the ELISA and the histochemical ICA test, repeatedly showed a lower diagnostic power in standardization programs for ICA compared with RIAs (39–41). Thus, the assay-dependent features of rGAD65ab D and E provide first examples for incongruent results in solid- and fluid-phase assays. They may allow us to understand better why autoantibodies detected in ELISA are less "disease-specific" than antibodies measured by RIA. In sum, the recombinant GAD65ab, rGAD65ab A–E, reflected well the natural autoantibody response in type 1 diabetes and suggest further use of the recombinant antibody technique for further insights in the humoral autoimmune response in type 1 diabetes.

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