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Human Monoclonal Antibodies Isolated from Type I Diabetes Patients Define Multiple Epitopes in the Protein Tyrosine Phosphatase-Like IA-2 Antigen

Verena Kolm-Litty,1*‡ Suzanne Berlo,1† Ezio Bonifacio,‡ Massimo Bearzatto,‡ Alfred M. Engel,† Michael Christie,§ Anette-G. Ziegler,∗ Thomas Wild,‡ and Josef Endl†

Protein tyrosine phosphatase-like IA-2 autoantigen is one of the major targets of humoral autoimmunity in patients with insulin-dependent diabetes mellitus (IDDM). In an effort to define the epitopes recognized by autoantibodies against IA-2, we generated five human mAbs (hAbs) from peripheral B lymphocytes isolated from patients most of whom had been recently diagnosed for IDDM. Determination and fine mapping of the critical regions for autoantibody binding was performed by RIA using mutant and chimeric constructs of IA-2- and IA-2β-regions. Four of the five IgG autoantibodies recognized distinct epitopes within the protein tyrosine phosphatase (PTP)-like domain of IA-2. The minimal region required for binding by three of the PTP-like domain-specific hAbs could be located to aa 777–979. Two of these hAbs cross-reacted with the related IA-2β PTP-like domain (IA-2β aa 741–1033). A further PTP-like domain specific hAb required the entire PTP-like domain (aa 687–979) for binding, but critical amino acids clustered in the N-terminal region 687–777. An additional epitope could be localized within the juxtamembrane domain (aa 603–779). In competition experiments, the epitope recognized by one of the hAbs was shown to be targeted by 10 of 14 anti-IA-2-positive sera. Nucleotide sequence analysis of this hAb revealed that it used a Vh germline gene (DP-71) preferably expressed in autoantibodies associated with IDDM. The presence of somatic mutations in both heavy and light chain genes and the high affinity or this Ab suggest that the immune response to IA-2 is Ag driven. The Journal of Immunology, 2000, 165: 4676–4684.

A
toimmunity directed against pancreatic islet cells causes progressive β-cell destruction and, as a consequence, insulin-dependent diabetes mellitus (IDDM).1 Although IDDM appears to be mostly a T cell-mediated autoimmune disease, the autoimmune process is accompanied by a humoral response against islet-specific Ags (1). Long before the onset of IDDM, a variety of islet-specific autoantibodies can be detected in the serum of affected individuals. Abs directed against islet cell Ags, such as glutamic acid decarboxylase (GAD65) (2), insulin (3), and the protein tyrosine phosphatase (PTP)-like proteins IA-2 (islet cell Ag 512) (4, 5) and IA-2β (phogrin/islet cell Ag-related PTP) (6, 7) are frequently present in the serum of prediabetic individuals and therefore represent valuable markers to predict IDDM in first degree relatives of IDDM patients. Anti-IA-2 autoantibodies are highly IDDM specific, and in combination with additional immunological markers such as anti-GAD65 and anti-insulin autoantibodies they are to date considered reliable markers for the diagnosis and prediction of IDDM (8).

IA-2 as a major target of humoral autoimmunity in IDDM was initially identified by screening of an islet cDNA expression library with patients’ sera (4). The full length coding sequence of the initial fragment, designated ICA512, was subsequently characterized and named IA-2 (9). IA-2 is closely related to phogrin/IA-2β, both being transmembrane proteins within the secretory granule membrane of neuroendocrine cells (7, 12). Their intracellular regions feature protein tyrosine phosphatase (PTP)-like domains of ~300 aa with no (IA-2) and weak (IA-2β) phosphatase activity detected (5, 13). IA-2 and IA-2β share 88% amino acid sequence homology within this region. The juxtamembrane domain of less than 100 aa links the PTP-like domain with the transmembrane domain and shows 50% homology between IA-2 and IA-2β. The luminal ectodomains of both proteins consist of ~600 aa and share less than 10% homology. Ab binding occurs within the intracellular cytoplasmic domain (11). IA-2 and IA-2β probably share common epitopes but also show distinct epitopes; 50–80% of IDDM sera that react with IA-2 also recognize IA-2β. Alternatively, even 95% of IDDM sera reacting with IA-2β also recognize IA-2 (10).

The role of autoantibodies in the IDDM autoimmune process remains elusive. The availability of disease-related human mAbs such as IA-2/IA-2β-specific Abs for immunological studies should help to elucidate the pathophysiological relevance of humoral components in the IDDM autoimmune process. Moreover, hAbs against IA-2 or IA2-β may help to overcome the difficulties of standardization of conventional IA-2 Ab assay systems and will allow comparison of different studies more reliably. However, all efforts to produce hAbs against IA-2 have failed thus far. In this
study, we describe for the first time the development and character-
ization of IA-2-specific hAbs of IgG isotype derived from pa-
tients with newly diagnosed IDDM. With the use of mutant forms of IA-2 and chimeric IA-2/IA-2β proteins, the epitopes targeted by these hAbs have been mapped in detail and Ig V-gene regions have been characterized.

Materials and Methods

Selection for IgG-positive cells and immortalization of B lymphocytes

Blood samples were obtained from the Institute of Diabetic Research, Mu-
 nich, Germany. PBMCs were isolated from heparinized peripheral blood of IDDM patients exhibiting high IA-2-specific Ab levels by Ficoll (Amer-
 sham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifuga-
tion (for details, see Table 1). To separate B lymphocytes by magnetic cell sorting, PBMCs were first labeled with mouse anti-human IgG Ab (Di-
anova, Hamburg, Germany) and subsequently with magnetic microbeads binding mouse IgG (Dynabeads M-280, Dionova). For immortalization, isolated IgG-positive B lymphocytes were incubated under regular gentle shaking for 2 h at 37°C with EBV-containing supernatant from the B-95-8 marmoset lymphoma cell line (American Type Culture Collection, Man-
asas, VA). B lymphocytes were seeded on microtiter plates at densities of 40–200 cells/well plus 20,000–50,000 irradiated PBMC feeder cells/well. Immortalized B lymphocytes were cultured in IMDM supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 400 μg/ml insulin, 1 mM pyruvate, nonessential amino acids, 1 μM o xoalacetate, and 100 U/ml IL-6 at 37°C and 7% CO 2 . Cells of this primary culture were fed once a wk and cultured over 2–3 wk before screening for IA-2-specific Abs.

Selection for anti-IA-2-producing cell lines and cloning of B cell lines

Supernatants of the EBV-transformed B lymphocyte cell lines were screened by a recently established anti-IA-2 ELISA (Roche Diagnostics, Penzberg, Germany). In brief, 50 μl supernatant of each well were diluted 1:2 with culture medium, transferred into streptavidin-coated microtiter plates (Microcost, Bernried, Germany), which had been coated with biotinyl-
ated conjugant recombinant IA-2ic from a concentration of 150 ng/ml (=2, 6 nM) and incubated for 1 h at room temperature while shaking (14). After exten-
tensive washing (three times) with PBS-0.05% Tween 20, 100 μl of mouse anti-human IgG peroxidase conjugate (0.2 U/ml, Roche) were added to the wells. After incubation (1 h at room temperature), plates were washed with PBS-0.05% Tween 20 (three times), and bound Abs were detected by re-
aaction with the peroxidase substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-
sulfonic acid) (ABTS, Roche). Extinction was measured after 1 h at 405 nm with a reference wavelength of 492 nm. As a standard, patients' sera were used to define critical amino acids.

Determination of heavy and light chain subclasses

Subclones of human IgG Abs were determined as follows. Anti IA-2 Abs bound to streptavidin-biotin-IA-2ic-coated microtiter plates (see above) were coupled to mouse anti-hAbs (10 μg/ml specific for 1) the different human IgG subclasses and 2) the two human light chains. The bound mouse Ab was detected by HRP-conjugated anti-mouse IgG (62.5 mM/ml (Roche) and ABTS reaction as described above.

Human IgG ELISA

Human IgG was determined by a sandwich ELISA, using goat anti-human IgG-Fc-specific Ab (Dianova) for coating of microtiter plates at a concentra-
tion of 10 μg/ml coating buffer (Roche), and bound human IgG was detected by 50 ng/ml peroxidase-coupled anti-human IgG-F(ab′)2-specific goat Ab (Dianova) and ABTS.

Determination of Ab affinity

Ab affinity was determined by real-time interaction analysis with a BIA-
core system (Pharmacia, Uppsala, Sweden) according to the manufactur-
er’s instructions. Biotinylated IA-2c was immobilized to streptavidin-
coated sensor chips.

Immunohistology on pancreas and other human tissues

To perform immune complexes with low background staining, IgG mAbs were complexed with HRP-conjugated goat anti-human IgG Abs as de-
scribed (15). Cryostat sections of human and animal pancreas and other human tissues were incubated with these preformed immune complexes for 2 h at 4°C in a humidified chamber. After intense washing of each section with cold PBS, the bound Ab was detected by staining with aminomethyl-
carbazole and hydrogen peroxide.

Double staining by indirect immunofluorescence

Double immunofluorescence staining was performed as follows. The iso-
lated IA-2-specific hAbs were labeled with digoxigenin (DIG, Roche) ac-
cording to the manufacturer’s instructions using a molar reaction mixture, Ab:DIG (1:15). Cryostat sections of human pancreas were incubated for 45 min with these DIG-labeled Abs (5 μg/ml) and the α cell-specific Ab BISL-32 (1:2000 diluted, Roche) (16). After three washings with PBS, the bound Abs were detected either by a FITC-conjugated mouse anti-DIG-Ab (diluted 1:600, Roche) and a Cy-3-conjugated goat anti-mouse IgG (diluted 1:200). The double-stained sections were examined under a fluorescence microscope.

In vitro translation of IA-2/IA-2β constructs

IA-2/IA-2β constructs used in this study are shown in Fig. 2. IA-2, 687–979 and IA-2β, 241–1033, were used to differentiate between the PTP-like domain of IA-2 and IA-2β. The IA-2β, 241–1033 served to identify Abs binding to the juxtamembrane region of IA-2. These constructs were prepared using the pGEM-T cloning vector (Promega, Madison, WI) under control of the SP6 promoter as described previously (11, 17). To define critical amino acids within epitope regions of the PTP domain, seven constructs with point mutations changing IA-2-specific amino acids to IA-β-specific amino acids were prepared using the pSP64 cloning vector (Promega) in combination with the QuickChange (Stratagene, La Jolla, CA) method (17). Additionally, two chimeric IA-2/IA-2β PTP constructs were prepared by in-frame joining of selected portions of IA-2 and IA-2β and expressed using pGEM-T vector as described recently (17). Purified plasmid DNA was transcribed, translated, and labeled using the TnT SP6-coupled rabbit reti-
culoocyte lysate system (Promega) in the presence of [35S]methionine (Amersham, Aylesbury, U.K.). Unincorporated radioactivity was removed by gel chromatography on NAP5 columns (Pharmacia).

Table 1. Immortalization of B lymphocytes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Duration of IDDM</th>
<th>Serum Anti-IA-2 Concentration (U)</th>
<th>IA-2-Positive Primary Wells</th>
<th>Monoclonal B Cell Lines</th>
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<td>10</td>
</tr>
<tr>
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<td>21</td>
<td>New onset</td>
<td>80</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>New onset</td>
<td>87</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>New onset</td>
<td>86</td>
<td></td>
<td>5</td>
</tr>
<tr>
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<td>76/12</td>
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<td>3</td>
<td>3</td>
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<td>2 yr</td>
<td>110</td>
<td>5</td>
<td>96/3, 96/4, 96/5</td>
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<td>101</td>
<td>3</td>
<td>103/5</td>
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<tr>
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<td>New onset</td>
<td>101</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>
Epitope mapping by RIA

The assay was essentially performed as described previously (17). Briefly, the in vitro translated protein fragments with 15,000–20,000 cpm incorporated radioactivity were diluted in 25 μl TBST (50 mM Tris, 150 mM NaCl (pH 7.2), 1% Tween 20), mixed with 2–10 μl hAb-containing cell supernatants, and incubated overnight on ice. Subsequently, immune complexes were recovered by adding 1 mg protein A-Sepharose (Pharmacia) preswollen and resuspended in 50 μl TBST, and incubated for 1 h at 4°C under shaking. After four washings with 2 ml TBST, the protein A-Sepharose-coupled immune complexes were resuspended in 100 μl TBST, transferred to a 96-well Optiplate (Packard, Groningen, The Netherlands), mixed with 150 μl Microscint 40, and cooled for 30 min under shaking. The precipitated radioactive was measured in a TopCount scintillation counter (Packard) for 5 min. Reactivity toward the mutagenized IA-2 proteins was evaluated by using a mouse anti-IA-2 mAb (mAb 76F9) as internal standard, which binds to an epitope within the juxtamembrane region of IA-2 (17).

Competition experiments using DIG-labeled anti IA-2 hAb

Different human anti-IA-2-reactive sera from recently diagnosed IDDM patients (kindly provided by P. Pozzilli, University Campus Biomedico, Rome, Italy) were used for blocking studies in an IA-2-specific ELISA performed as described above with some modifications. After coupling of biotinylated IA-2 (150 μg/ml) to streptavidin-coated microwell plates, 100 μl of IA-2-reactive sera (diluted to 125 ng/ml IA-2-specific Ab equivalents) were transferred to the wells and incubated for 1 h at room temperature to block epitope-specific binding sites. After intense washing with PBS-0.05% Tween 20, the remaining free binding sites were detected by monoclonal DIG-labeled anti-IA-2 Ab (2.5 ng/ml). Bound DIG-labeled hAb was detected by HRP-conjugated mouse anti-DIG Ab (1:1000 diluted, Roche) and ABTS reaction. ODs were measured at 405 nm with a reference wavelength of 492 nm.

Cloning of human Ab fragments

RNA isolation, cDNA synthesis, and RT-PCR amplification (primary PCR).

Total RNA from ~10^6 lymphoblastoid B cells producing IA-2-specific hAb was isolated using the Trisolv method (Biotech, Houston, TX) according to the manufacturer’s instructions and subjected to cDNA synthesis using an AMV Reverse Transcriptase Kit (Roche). Primary PCR was performed as described by McCafferty et al. (18) using six BACK VH and four FOR J{\textsubscript{H}} gene family-specific primers for the variable region of heavy IgG chains, seven BACK V{\textsubscript{H}} and four FOR J{\textsubscript{H}} specific primers for the variable region of light chains, and six BACK V{\textsubscript{L}}- and five FOR J{\textsubscript{L}}-specific primers for the variable region of κ light chains. For amplification of heavy and light chain cDNA fragments, each BACK primer was used in a separate reaction mixture. A 50 μl reaction mixture was prepared containing 1× PCR buffer with 20 mM Mg{\textsuperscript{2+}}, 5 mM PCR nucleotide mix, 5 μM primer, and 0.1–0.75 U Pwo polymerase (Roche), 0.5 μM primer, and 0.1–0.75 μL template DNA. The reaction mixture was subjected to 30 cycles of amplification, 1 cycle consisting of 1 min denaturation at 94°C, 1 min touchdown-annealing at 70–40°C, and 1 min extension at 72°C. Amplification products were analyzed on a 1.4% agarose gel and purified by gel extraction (Qiagen, Hilden, Germany).

Linker and assembly PCR to generate single-chain variable fragments (scFv).

A commercially available mouse DNA fragment (Pharmacia) containing 1× endonuclease IgM restriction sites, the scFv fragments were ligated into pCANTAB-5E-phagemid (Pharmacia) for sequence determination and expression of soluble scFv-Abs (for details, see manufacturer’s instruction manual).

Sequencing

Amplified cDNA fragments and recombinant phagemid DNA were sequenced by the dideoxy chain termination procedure (19). The established V{\textsubscript{H}}, V{\textsubscript{L}}, D{\textsubscript{H}}, and J{\textsubscript{H},} sequences, and J{\textsubscript{H},} sequences were compared with sequences present in the V BASE sequence directory (www.nrc-spe.ca.ac.uk/int-doc/public/INTRO.html). Subdivision of sequences into framework (FR) and complementarity-determining regions (CDRs) were performed according to the method of Kabat (20).

Results

Generation of hAbs

B lymphocytes from 11 IDDM patients exhibiting high anti-IA-2 Ab levels in their sera were subjected to EBV immortalization. By selecting IgG-producing B lymphocytes, we eliminated the IgM-producing B lymphocytes, which predominate in the pool of PBLs and usually produce Abs of low affinity and specificity (21). Screening of ~40,000 B lymphocyte culture supernatants by an anti-IA-2-specific ELISA identified 16 anti-IA-2 Ab-producing B lymphocyte cell lines (Table I). Five of them could be stabilized at single-cell level by repeated limiting dilution. Due to the instability and low cloning efficiency of EBV-transformed B cell lines (22) 11 of the primarily anti-IA-2-positive cultures did not survive this procedure or stopped Ab production during the cloning steps. Addition of IL-6 (100 U/ml) as a B cell growth factor (23) ameliorated the survival rate but could not rescue most of the unstable cell lines.

Five cell lines producing hAb anti-IA-2 were derived from three different patients who showed very high anti-IA-2 Ab sera levels (Table I): cell line 76/12 (patient 6); cell lines 96/3, 96/4, and 96/5 (patient 9); and cell line 103/5 (patient 10). Nevertheless, only one cell line (96/3) showed long term stability for >12 mo and could be used as constant source of anti-IA-2 Ab with a production rate of 5–8 μg/10^6 cells in 24 h. This hAb was purified and its affinity was determined by the BLAcore method. It revealed high affinity to recombinant IA-2ic which was coupled to the sensor chip (K{\textsubscript{D}} = 0.13 nM). Using the hAb{\textsubscript{96/3}} as standard in the anti IA-2-ELISA proved that the assay was linear for Ab concentrations within 0.5–5 ng/ml.

Subclass distribution of IA-2-specific hAbs

Analysis of the IgG subclasses classified all five isolated hAbs as IgG1, which is in accordance with the observation that in IDDM patients pancreas-reactive autoantibodies are predominantly IgG1 (24, 25). Three of the light chains expressed were of κ subtype and two of the light chains were of λ subtype.

Tissue and species specificity of IA-2-specific hAbs

The immunohistochemistry on pancreata of different species revealed no species specificity for human, mouse, and rat islets. All tested hAb anti-IA-2 stained the pancreatic islets, whereas exocrine tissue showed no reactivity (Fig. 1A). Background staining by endogenous Ig in tissues was avoided by using preformed (anti-IA-2-peroxidase-anti-human IgG) complexes. Double staining of pancreatic islets with DIG-labeled hAb{\textsubscript{96/3}} and the κ cell-specific mouse mAb BISL-32 showed that this IA-2-specific hAb stained β cells and a majority of the α cells (Fig. 1B). Among other human tissues tested, IA-2-specific hAbs revealed no reactivity toward thyroid, liver, lung, stomach, renal, or intestine tissue. However, a faint reactivity could be seen with neurons of the cerebellar cortex.

Epitope studies

For epitope studies, we used 13 different constructs of IA-2/IA-2b (Fig. 2) in a conventional anti-IA-2 RIA. These constructs comprised various truncated forms of IA-2 (PTP domain only, PTP domain plus juxtamembrane region, and a construct including also the transmembrane region). In addition, different mutated forms of IA-2 as well as chimeras between IA-2 and IA-2b were tested.

Four of five anti-IA-2 autoantibodies, hAb{\textsubscript{96/12}}, hAb{\textsubscript{96/3}}, hAb{\textsubscript{96/4}}, and hAb{\textsubscript{96/5}} reacted with IA-2{\textsubscript{687–979}}, the PTP-like domain of IA-2, and did not react with IA-2{\textsubscript{389–779}} covering the juxtamembrane region of IA-2 (Table II). All four hAbs thus recognized epitopes...
within a region between aa 687 and aa 979. In addition, hAb 96/3 revealed a strong reactivity toward construct IA-2 687–979, and one further hAb reacted against the juxtamembrane region of IA-2 or a C-terminally closely neighboring region. As expected, none of the isolated Abs was exclusively IA-2β specific because the ELISA screening system was performed with IA-2ic.

To define epitope regions within the PTP-like domain in more detail, different mutant and chimeric constructs, as well as the construct IA-2 687–979 were used (Fig. 2 and Table II). One of the IA-2 PTP domain-specific Abs, hAb 96/4, did not bind to construct IA-2 687–979 but showed reactivity with construct IA-2 687–979. Obviously, the Ab recognized an epitope within aa 687–979 with residues 687–777 being critical for Ab binding. The Ab reacted with all the mutant constructs except construct T804V. Therefore, residue 804 is likely to be required for the tertiary structure of the epitope or is a critical Ab contact site. There was no reactivity of hAb 96/4 toward any of the chimeric constructs containing IA-2 regions 777–889 framed by regions derived from the IA-2β isoform, indicating that IA-2-specific amino acids outside the region 777–889 were involved in Ab binding.

The second IA-2 PTP domain-specific Ab, hAb 96/5, revealed reactivity to construct IA-2 687–979 and also bound to construct IA-2 741–1033; therefore, its epitope was located within the region between residues 777 and 979. This Ab did not react with the chimeric constructs; therefore, hAb 96/5 binding required residues 777–794 and/or residues 889–979. All mutagenized constructs reacted with hAb 96/5 except the one in which positions 876, 877, 878, and 880 were simultaneously exchanged. Obviously, the region between aa 876 and 880 contributed to epitope formation while the exchange of IA-2-specific aa 804, 813, 821, 822, 862, and 886 did not affect Ab binding.

The hAbs cross-reactive with IA-2β (hAb 96/3 and hAb 76/12) revealed a similar reaction pattern toward all chimeric and mutagenized constructs (Table I). Both hAbs reacted with constructs IA-2 687–979 and IA-2-β 741–1033, with hAb 76/12 exhibiting much weaker reactivity (320 cpm vs 3600 cpm with hAb 96/3). Because

FIGURE 1. A, Immunohistology of human pancreas stained with anti-IA-2 hAbs. Cryostat sections of human pancreas were incubated with preformed immune complexes composed of peroxidase-conjugated anti-human IgG and hAb 96/3. Detection was performed with aminoethylcarbazole and hydrogen peroxide. B, Double staining of human pancreas. α cells were stained with the α cell-specific BISL-32 mouse mAb and Cy-3-conjugated anti-mouse IgG. DIG-labeled IA-2-specific hAb 96/3 was detected by FITC-conjugated anti-DIG Ab.

FIGURE 2. IA-2 and IA-2β cDNA constructs used in this study. Numbers indicate the amino acid positions within the predicted protein sequences of IA-2 (GenBank accession number L18983) and IA-2β (GenBank accession number Y08569). JM, Juxtamembrane region; TM, transmembrane region.
both hAbs did not react with construct IA-2\textsubscript{389–779}, residues within region 777–979 were compulsory to form the epitopes. Because there was strong binding of both hAbs toward construct IA-2\textsubscript{777–799}, a conformational contribution of amino acids N-terminal to this region could be excluded. Determination of the exact binding region of hAb\textsubscript{76/12} was possible due to its weak reaction toward IA-2\textsubscript{889–979} with potential participation of IA-2\textsubscript{845} and Juxtamembrane domain, because there was no significant loss of reactivity outside this region. There was no significant loss of reactivity toward any of the mutant constructs; therefore, IA-2-specific aa 804, 813, 821, and 822 located within this region seemed not to be critical for Ab binding of hAb\textsubscript{76/12}. The epitope of hAb\textsubscript{96/3} could not be restricted to a smaller region than aa 777–979 because hAb\textsubscript{96/3} was highly cross-reactive and all chimeric and mutant constructs revealed strong reactivity with this Ab.

**Competition experiments**

The in vivo relevance of the epitope recognized by hAb\textsubscript{96/3} was tested by ELISA blocking studies using 14 different anti-IA-2-reactive sera of newly diagnosed IDDM patients. In a competition assay, 100% inhibition was achieved after blocking 2.5 ng/ml DIG-labeled IA-2-specific hAb\textsubscript{96/3} with 125 ng/ml unlabeled hAb\textsubscript{96/3}. Therefore, IDDM sera for blocking studies were diluted to a concentration of 125 ng/ml IA-2-specific Ab equivalents as determined in the conventional anti-IA-2 ELISA. Ten of 14 tested sera competed with hAb\textsubscript{96/3}, indicating that they contained anti-IA-2-specific Ab(s) recognizing the same or an adjacent epitope as hAb\textsubscript{96/3}.

**Sequence analysis of hAb\textsubscript{96/3}**

We determined the nucleotide sequence of hAb\textsubscript{96/3} (Fig. 3) by cloning of the V\textsubscript{H} and V\textsubscript{L} DNA fragments as scFv Abs. Soluble scFv Abs expressed in Escherichia coli bound specifically to IA-2\textsubscript{a}, which was confirmed by ELISA (data not shown). Gene sequencing was performed on two independently generated PCR products using primers annealing within different gene segment regions to exclude that the observed mutations were not generated by the gene amplification and sequencing process.

Alignment of the V\textsubscript{H} gene sequence of hAb\textsubscript{96/3} against the V\textsubscript{H} base yielded V\textsubscript{H}\textsubscript{4} as germline counterpart. HAb\textsubscript{96/3} displayed the highest degree of identity (86%) with germline V\textsubscript{H}\textsubscript{4 DP-71 gene (Fig. 3, Table IV). The 39 nucleotide differences were scattered throughout FRs and CDRs and yielded amino acid replacement-silent mutation ratios (R:S ratios) of 0.5 and 0.9, respectively. The D segment sequence of hAb\textsubscript{96/3} revealed a high homology to gene segment D6–25 over a stretch of 19 positions plus 8 additional nucleotides at the 5’-end (Fig. 3A). Comparison of the expressed J\textsubscript{H} gene sequence with those of the known germline J\textsubscript{H} genes showed that hAb\textsubscript{96/3} used a slightly mutated form of J\textsubscript{H}4 that bound to the epitope contained within the juxtamembrane region of IA-2.

**Table II. Reactivity of IA-2-specific hAbs toward different IA-2/IA-2B constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>hAb\textsubscript{76/12}</th>
<th>hAb\textsubscript{96/3}</th>
<th>hAb\textsubscript{96/4}</th>
<th>hAb\textsubscript{96/5}</th>
<th>hAb\textsubscript{103/5}</th>
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<td>IA-2\textsubscript{603–979}</td>
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<td>IA-2\textsubscript{687–979}</td>
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<td>+</td>
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<td>IA-2\textsubscript{777–799} (juxtamembrane)</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>IA-2-\textsubscript{3841–1033}</td>
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<td>+</td>
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<td>–</td>
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<tr>
<td>Minimal region identified for Ab binding</td>
<td>IA-2\textsubscript{777–799} or IA-2-\textsubscript{3841–1033}</td>
<td>IA-2\textsubscript{777–799} or IA-2-\textsubscript{3841–1033}</td>
<td>IA-2\textsubscript{687–979}</td>
<td>IA-2\textsubscript{777–979}</td>
<td>IA-2\textsubscript{963–979}</td>
</tr>
<tr>
<td>IA-2-\textsubscript{741–1033} PTP domain</td>
<td>IA-2-\textsubscript{741–1033} PTP domain</td>
<td>IA-2-\textsubscript{741–1033} PTP domain</td>
<td>PTP domain</td>
<td>PTP domain</td>
<td>PTP domain</td>
</tr>
<tr>
<td>Critical amino acids identified</td>
<td>IA-2\textsubscript{794–845} Position 815 and/or 818, 829, 830, 834</td>
<td>None identified</td>
<td>IA-2\textsubscript{687–777} Position 804</td>
<td>IA-2\textsubscript{777–793} and/or IA-2\textsubscript{849–979} Positions 876, 877, 878, 880</td>
<td>None identified</td>
</tr>
</tbody>
</table>

* Reactivity is expressed relative to a 1:10 dilution of a pool of human normal serum. Threshold reactivity, expressed as “+”, is at least 5-fold above the reactivity observed with the 1:10 diluted human serum, which measured in the range of 30–80 cpm. Reactivity against the mutagenized IA-2 proteins is expressed relative to that observed with a mouse anti-IA-2 mAb (76F) that binds to an epitope contained within the juxtamembrane region of IA-2.
Alignment of hAb96/3 Vκ nucleotide sequence displayed the highest degree of identity (93%) with the germline IGLV3S2 gene of the Vκ3 family (Fig. 3, Table IV). The nucleotide differences resulted in higher putative R:S ratios within the CDR than the FR, being 2.3 and 1.0, respectively. More than one-half of the nucleotide changes were replacement changes. The Jκ segment of hAb 96/3 exhibited two silent mutations and a stretch N-terminal additions coding for four amino acids within the CDR3 region (Fig. 3).

Discussion
The processes of humoral immune response leading to production of islet cell autoantibodies in IDDM are largely unknown. The disease is believed to be mainly due to a cytotoxic immune response against islet cell Ags, resulting in pancreatic β cell destruction. Recent evidence, however, indicates that B lymphocytes play a critical role within the autoimmune process given that B lymphocyte-deficient mice are free of insulinitis and diabetes (26). A variety of islet cell autoantigens is recognized by autoreactive T and B lymphocytes. Autoantibodies specific for self-Ags arise long before the clinical onset of IDDM and provide valuable markers for disease prediction. They may serve to investigate the autoimmune process to define autoantigens being attacked and to determine epitope structures.

Our studies focused on generating human mAbs directed against the diabetes-associated autoantigen PTP-like IA-2 to perform a detailed mapping of IA-2 epitopes recognized by autoreactive B lymphocytes present in IDDM patients. In studies performed with sera from IDDM patients, it has been previously described that the cytoplasmic portion of IA-2 (IA-2ic) is the major target of humoral autoimmunity. Sera react against either the juxtamembrane region or the PTP-like domain of IA-2ic, and there is no binding of autoantibodies to the IA-2 ecto (luminal)- and transmembrane domain (11, 27). Therefore, the complete cytoplasmic IA-2ic part was sufficient for screening of anti-IA-2 Ab producing B cell lines.

By combining EBV transformation with a high throughput ELISA screening system, we succeeded in generating five human B cell lines producing anti-IA-2-specific mAbs (hAb76/12, hAb96/3, hAb96/4, hAb96/5, and hAb103/5) from peripheral blood of three IDDM patients exhibiting very high anti-IA-2 reactivity. To generate only IgG-producing B cell lines, we preselected IgG-positive B cells before EBV transformation as described by Richter et al. (28). All isolated B cell lines produced anti-IA-2 Abs of the IgG class. Four of the human B cell lines maintained stable Ab production for several months; only one line (96/3) showed stability for >1 year with a production rate of 5–8 μg/10^6 cells in 24 h. The generation of stable B cell lines for production and analysis of human autoantibodies is still not a routine procedure such as the generation of murine hybridomas. In the context of autoimmune diabetes to date, only human B cell lines secreting autoantibodies against insulin (29) and glutamate decarboxylase (28) have been described. To our knowledge, this is the first report describing hAbs against IA-2. These hAbs should greatly facilitate a detailed mapping of the autoantigenic epitopes within the IA-2ic sequence. We performed epitope mapping studies using in a first step a series of truncated forms of IA-2 and IA-2β. These studies revealed that the five isolated mAbs recognized distinct epitopes. Two hAbs were directed against the PTP-like domain of IA-2 and IA-2β, two hAbs were restricted toward the PTP-like domain of IA-2 and one hAb was reactive against the juxtamembrane region, which is unique to IA-2 (11). Fine mapping was performed by using mutant and chimeric constructs of IA-2 and IA-2β. The epitopes of the cross-reactive anti-IA-2/IA-2β autoantibodies could be limited to a region between residues 777 and 979, which includes the most conserved region among various PTPs between residues 777 and 937 (30). Cross-reactivity is likely to occur within this conserved region. Critical residues for the epitope of the weakly cross-reactive hAb96/13 were localized in the region between residues 794 and 864. Mutagenization of the IA-2-specific residues 804, 813, 821, and 822 to the corresponding IA-2β-specific residues did not change reactivity of this mAb. Presumably, the remaining IA-2-specific residues 815, 818, 829, 830, and 834 were responsible for the higher reactivity toward IA-2 than toward IA-2β. The epitope recognized by hAb96/3 could not be identified with specific residues within the region 794–845, because there was equally strong reactivity between the two isoforms and none of the mutated positions influenced Ab binding. A cross-reactive epitope region located in the PTP domain between residues 687 and 979 has already been described (17). Our results demonstrate that at least two different epitopes exist within the PTP-like domain of IA-2 which can induce Abs cross-reactive with the IA-2β isoform.

The two IA-2-specific Abs also recognized different epitopes. One was located within the PTP-like domain requiring aa 687–979. Although Lampasona et al. (11) already described this region being targeted by IDDM sera autoantibodies, fine mapping with hAb96/3 revealed that region 687–777 and the IA-2-specific residue 804 play a critical role for epitope formation. Because none of the chimeric constructs reacted with this Ab, IA-2-specific amino acids outside the IA-2-region 794–889 were also important for Ab...
binding. For the epitope recognized by the second IA-2-specific Ab (hAb 96/5), aa 889–979 at the C terminus of IA-2 were required. According to Lampasona et al. (11), most IDDM patients have Abs directed against the region between 777 and 937 of the PTP-like domain. Four of our five mAbs recognized epitopes within or very close to this described epitope region. In addition, competition studies performed with the PTP-like domain specific hAb 96/3 showed that it could be very efficiently competed by patients’ sera.

FIGURE 3. Alignment of the nucleotide sequences (A) and the amino acid sequences (B) of germline V-genes DP-71 and IGLV3S2 (top line) and hAb96/3 V genes (bottom line). Dashes represent identity between both sequences. Solid lines above each cluster encompass FR and CDR regions. PCR primer sites are underlined. The presented V sequences are deposited in the V BASE directory under GenBank accession numbers Z12371 (VH), X97051 (DH), X86355 (JH), X71966 (VL), and M15641 (JL).
demonstrating that an identical or a closely adjacent epitope was targeted by serum Abs induced in a majority of patients.

Immunohistochemistry studies demonstrated that none of the IA-2-specific autoantibodies reacted with thyroid, liver, lung, stomach, renal, or intestine tissues, but all five reacted with pancreatic islets of different mammalian species and with neurons of the cerebellar cortex. IA-2 and also IA-2β are predominantly expressed in cells of neuroendocrine origin, particularly in pancreatic islets and brain (10). Double staining by the PTP domain-specific hAb96/3 showed that this Ab reacted with pancreatic β cells and also with α cells. Lu et al. (31) found a differential expression of IA-2 and IA-2β in pancreatic cell lines. IA-2 was preferentially expressed in the α cell line, whereas IA-2β was preferentially expressed in a β cell line. The fact that hAb96/3 is cross-reactive with IA-2β would explain why it stained both α cells and β cells.

Analysis of the nucleotide sequences of the anti-IA-2-specific hAb96/3 revealed that this Ab reacted with pancreatic β cells and also with α cells. Lu et al. (31) found a differential expression of IA-2 and IA-2β in pancreatic cell lines. IA-2 was preferentially expressed in the α cell line, whereas IA-2β was preferentially expressed in a β cell line. The fact that hAb96/3 is cross-reactive with IA-2β would explain why it stained both α cells and β cells.

Table IV. Gene analysis of hAb96/3

<table>
<thead>
<tr>
<th>V H Family</th>
<th>Closest V H Gene</th>
<th>% Nucleotide Identity</th>
<th>Number of Silent and Replacement Substitutions*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FR1</td>
</tr>
<tr>
<td>Heavy chain</td>
<td>V H 4</td>
<td>DP-71</td>
<td>86 (S)</td>
</tr>
<tr>
<td>Light chain</td>
<td>V L 3</td>
<td>IGLV3</td>
<td>84 (R)</td>
</tr>
</tbody>
</table>

* Nucleotide substitutions within the primer regions are not included in calculation.

FIGURE 3. (continued)
consequence R:S values become blurred. However, the presence of mutations observed in both \( V_\text{H} \) and \( V_\text{L} \) gene segments and the high affinity of this Ab (K\(_D\) 0.13 nM) are clearly indicative for an Ag-driven selection process.

In summary, we generated five human monoclonal anti-IA-2 specific IgG Abs directed against five distinct epitope regions of IA-2ic. All of these were conformational epitopes that required larger protein domains for binding, suggesting that native folded IA-2 is the immunogen for autoreactive B-cells. Whereas most of the epitopes clustered in the PTP-like domain, one epitope could be localized to the juxtamembrane region. From one of the PTP domain-specific hAbs the V-genes could be characterized. It used a \( V_\text{L} \) germline gene already described to be overrepresented in autoantibodies associated with IDDM. Using this hAb for competition studies, it was shown that most IDDM patient sera contained high amounts of autoantibodies recognizing an identical or a closely adjacent epitope. Because this hAb can be provided in unlimited amounts, it should be of great value for development and standardization of anti IA-2 screening assays for prediction of IDDM.

Acknowledgments

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References


29. Ikenatsus, J., H. Ichiyoshii., E. W. Schettino, M. Nakamura, and P. Casali. 1994. \( V_\text{H} \) and \( V_\text{L} \) motif analysis and Dr. L. Mantovani (Roche Diagnostics) for discussion and for critically reading the manuscript.