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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,† Suzanna Berlo,‡ 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 V_{H} 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.
study, we describe for the first time the development and characterization of IA-2-specific hAbs of IgG isotype derived from patients 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, Munich, Germany. PBMCs were isolated from heparinized peripheral blood of IDDM patients exhibiting high IA-2-specific Ab levels by Ficoll (Ameri-

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 (Dianova, Hamburg, Germany) and subsequently with magnetic microbeads binding mouse IgG (Dynabeads M-280, Dianova). For immobilization, 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 marrow lymphoma cell line (American Type Culture Collection, Man-

assas, 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 

μg/ml streptomycin, 10% FCS, 2 mML-glutamine, 100 U/ml penicillin, 100 

μg/ml streptomycin, 400 

μM insulin, 1 mM pyruvate, nonessential amino acids, 1 μM oxaloacetate, and 100 U/ml IL-6 at 37°C and 7% CO2. 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 (Microcoat, Bernried, Germany), which had been coated with biotinyl-

ated recombinant IA-2cic at a concentration of 150 ng/ml (=2, 6 nM) and incubated for 1 h at room temperature while shaking (14). After ex-
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-

action 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 proved to be positive for anti-IA-2 Ab in conventional RIA were used.

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

IA-2/IA-2β constructs used in this study are shown in Fig. 2. IA-2b87–979 and IA-2βb41–1032, were used to differentiate between the PTP-like domain of IA-2 and IA-2β. The IA-2b87–279 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 reticulocyte 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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<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>New onset</td>
<td>86</td>
<td>2</td>
<td>76/12</td>
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<td>32</td>
<td>New onset</td>
<td>87</td>
<td>3</td>
<td>103/5</td>
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<tr>
<td>5</td>
<td>33</td>
<td>New onset</td>
<td>86</td>
<td>3</td>
<td>103/5</td>
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<tr>
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<td>9</td>
<td>New onset</td>
<td>184</td>
<td>3</td>
<td>76/12</td>
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<td>19</td>
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<tr>
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<td>33</td>
<td>2 yr</td>
<td>78</td>
<td>3</td>
<td>103/5</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>2 yr</td>
<td>110</td>
<td>5</td>
<td>96/3, 96/4, 96/5</td>
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<tr>
<td>10</td>
<td>49</td>
<td>New onset</td>
<td>101</td>
<td>3</td>
<td>103/5</td>
</tr>
<tr>
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<td>101</td>
<td>3</td>
<td>103/5</td>
</tr>
</tbody>
</table>
Epitope Characterization of Human Anti-IA-2 Autoantibodies

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) to the cell supernatants and incubated for 1 h at 4°C. 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 radioactivity 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 76F) as internal counter (Packard) for 5 min. ODs were measured at 405 nm with a reference wavelength of 500 nm.

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_d = 0.13 nM). Using the hAb96/3 as standard in the anti IA-2-ELISA proved that the assay was linear for Ab concentrations within 0.1–5 ng/ml.

Subclass distribution of IA-2-specific hAbs

Analysis of the IgG subtypes 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 against-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 hAb96/3 and the α 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 (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-2 were tested.

Four of five anti-IA-2 autoantibodies, hAb96/12, hAb96/3, hAb96/4, and hAb96/5 reacted with IA-2(687–979), the PTP-like domain of IA-2, and did not react with IA-2(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-2b 741–1033, the IA-2b-PTP-like domain. Neither hAb 96/4 nor hAb 96/5 reacted toward the IA-2b-PTP-like domain, whereas hAb 76/12 showed weak reactivity. A particular epitope could be specified for hAb 103/5. It recognized IA-2 389–779 but not the partially overlapping IA-2 687–979 region and therefore must be directed against a determinant located N-terminal to the PTP-like domain. Because all mAbs were initially screened with the truncated construct IA-2ic 603–979, we suppose that hAb 103/5 is directed against a region within or very close to the juxtamembrane region of IA-2. In summary, we have isolated two hAbs recognizing one or two determinants that are shared within the PTP-like domain of IA-2 and IA-2b, two hAbs with restricted specificity toward the PTP-like domain of IA-2, and one further hAb reactive against the juxtamembrane region of IA-2 or a C-terminally closely neighboring region. As expected, none of the isolated Abs was exclusively IA-2b 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-2 777–979, 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-2 777–979 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 794–845 and 794–889 framed by regions derived from the IA-2b isoform, indicating that IA-2-specific amino acids outside the region 794–889 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-2 777–979; 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-2b (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-2b 741–1033, with hAb 76/12 exhibiting much weaker reactivity (320 cpm vs 3600 cpm with hAb 96/3). Because
both hAbs did not react with construct IA-2\textsubscript{389–779}, residues within region 779–979 were compulsory to form the epitopes. Because there was strong binding of both hAbs toward construct IA-2\textsubscript{777–979}, a conformational contribution of amino acids N-terminal to this region could be excluded. Determination of the exact binding region of IA-2\textsubscript{794–845} with potential participation of IA-2/IA-2\textsubscript{b}741–1033 revealed strong reactivity with this Ab. The in vivo relevance of the epitope recognized by hAb 96/3 was shown by ELISA blocking studies using 14 different anti-IA-2 constructs. The minimal region identified for Ab binding was IA-2\textsubscript{777–979} or IA-2\textsubscript{777–979} PTP domain. Critical amino acids identified were IA-2\textsubscript{794–845} Position 815 and/or 818, 829, 830, 834.

### Table II. Reactivity of IA-2-specific hAbs toward different IA-2/IA-2\textsubscript{b} 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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<tr>
<td>IA-2\textsubscript{603–979}</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>IA-2\textsubscript{687–979}</td>
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<tr>
<td>IA-2\textsubscript{777–979}</td>
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<td>+++</td>
<td>–</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>IA-2\textsubscript{741–1033}</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IA-2-\textsubscript{b}741–1033</td>
<td>+</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</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.

Percent inhibition ranged between 20 and 85% (Table III).
Alignment of hAb96/3 Vκ nucleotide sequence displayed the highest degree of identity (93%) with the germline IGLY3S2 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 hAb96/3 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 B and T 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 hAb105/3) 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 Abs against IA-2. These Abs 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 hAb96/12 were localized in the region between residues 794 and 845. 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 isofrom.

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 (hAb96/3), 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 hAb96/3 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.

Analysis of the nucleotide sequences of the anti-IA-2-specific hAb96/3 revealed that the Ab used a gene of the Vh4 family in association with the Va3 gene. The highest homology for the Vh gene was found with germline gene DP-71, which has already been reported to be preferably rearranged in GAD-specific hAbs. Richter et al. (32) reported the sequence analysis of seven anti-GAD65 hAbs and found that three of them used a member of the Vh4 family. Two of them derived from the same germline gene as our IA-2-specific hAb96/3. Moreover, another two anti-GAD-specific hAbs described by Madec et al. (33) used a member of the Vh4 family, one of them being germline gene DP-71. Thus, the Vh4 family and especially the DP-71 germline gene seem to be overrepresented in autoantibodies associated with autoimmune diabetes. Comparison of the Vh gene sequence between DP-71 and hAb96/3 revealed that hAb96/3 accumulated a series of nucleotide exchanges. These mutations were scattered throughout the CDR (22 mutations) and FR (17 mutations). Eight mutations within the CDR and seven mutations within the FR resulted in amino acid replacements. The resulting R:S ratio within CDR was 0.9 and 0.5 within the FR, which is relatively low compared with V genes of other high affinity Abs and autoantibodies (34). Also the VL segment of hAb96/3 displayed a moderate R:S ratio (2.3) throughout the CDR and, as expected, a low R:S ratio (1.0) in the respective FR. The relatively low R:S ratios found in the VH and VL segment may be explained in at least two ways: 1) what we considered as silent mutations could in fact derive from the existence of allelic VH or VL gene variants used by this patient; and 2) a number of nonrelevant silent mutations may accumulate in the V gene regions during a chronic autoimmune disease like type I diabetes, and as a

![Diagram of Vh Segment](image.png)

**FIGURE 3. (continued)**

<table>
<thead>
<tr>
<th>Vh Family</th>
<th>Closest Gene</th>
<th>% Nucleotide Identity</th>
<th>Number of Silent and Replacement Substitutions*</th>
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<th>Closest Jh Gene</th>
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<tr>
<td>Heavy chain</td>
<td>Vh4</td>
<td>DP-71</td>
<td>86 (S)</td>
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<td></td>
<td></td>
<td>84 (R)</td>
<td>3 3 0 5 4</td>
<td>(0.5)</td>
</tr>
<tr>
<td>Light chain</td>
<td>Va3</td>
<td>IGLV3</td>
<td>93 (S)</td>
<td>1 0 2 2 2 1</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s2</td>
<td>87 (R)</td>
<td>1 3 2 3 2 1</td>
<td>(1)</td>
</tr>
</tbody>
</table>

* Nucleotide substitutions within the primer regions are not included in calculation.
consequence R:S values become blurred. However, the presence of mutations observed in both V\textsubscript{H} and V\textsubscript{L} gene segments and the high affinity of this Ab (K\textsubscript{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\textsubscript{H} 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.

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References