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Human monoclonal islet cell antibodies from a patient with insulin-dependent diabetes mellitus reveal glutamate decarboxylase as the target antigen

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ABSTRACT **The autoimmune phenomena associated with destruction of the β cell in pancreatic islets and development of type 1 (insulin-dependent) diabetes mellitus (IDDM) include circulating islet cell antibodies. We have immortalized peripheral blood lymphocytes from prediabetic individuals and patients with newly diagnosed IDDM by Epstein–Barr virus transformation. IgG-positive cells were selected by anti-human IgG-coupled magnetic beads and expanded in cell culture. Supernatants were screened for cytoplasmic islet cell antibodies using the conventional indirect immunofluorescence test on cryostat sections of human pancreas. Six islet cell-specific B-cell lines, originating from a patient with newly diagnosed IDDM, could be stabilized on a monoclonal level. All six monoclonal islet cell antibodies (MICA 1–6) were of the IgG class. None of the MICA reacted with human thyroid, adrenal gland, anterior pituitary, liver, lung, stomach, and intestine tissues but all six reacted with pancreatic islets of different mammalian species and, in addition, with neurons of rat cerebellar cortex. MICA 1–6 were shown to recognize four distinct antigenic epitopes in islets. Islet cell antibody-positive diabetic sera but not normal human sera blocked the binding of the monoclonal antibodies to their target epitopes. Immunoprecipitation of ^{35}S-labeled human islet cell extracts revealed that a protein of identical size to the enzyme glutamate decarboxylase (EC 4.1.1.15) was a target of all MICA. Furthermore, antigen immunotrapped by the MICA from brain homogenates showed glutamate decarboxylase enzyme activity. MICA 1–6 therefore reveal glutamate decarboxylase as the predominant target antigen of cytoplasmic islet cell autoantibodies in a patient with newly diagnosed IDDM.**

Autoimmunity directed against pancreatic islet cells results in slowly progressing β-cell destruction preceding the clinical onset of insulin-dependent diabetes mellitus (IDDM). The autoimmune phenomena associated with the disease include lymphocytic infiltration of the islets and circulating serum antibodies to various islet-specific antigens (1, 2). Among the diabetes-associated autoantibodies, two major specificities have been described: cytoplasmic islet cell antibodies (ICA), staining cytoplasmic islet cell components by indirect immunofluorescence on sections of human pancreas (3), and the 64-kDa antibodies, precipitating a 64-kDa protein from islet cell extracts (4). Whereas 64-kDa antibodies were shown to be β-cell specific within the islet (5), some ICA-positive sera have been described to react with all islet cells (3, 6). The target antigen of the 64-kDa antibodies was recently identified as the enzyme glutamate decarboxylase (GAD; EC 4.1.1.15) (7). The cytoplasmic molecules recognized by ICA were, however, suggested to be gangliosides rather than proteins (8, 9), although a defined target antigen of ICA has not been identified.

Disease-related monoclonal autoantibodies from patients with IDDM would greatly facilitate isolation and characterization of the target antigens of ICA and help to clarify their possible role in the autoimmune process. However all efforts to produce human monoclonal antibodies with ICA characteristics from patients with IDDM have been unsuccessful so far (10–12). In this study we describe the development and characterization of six stable IgG ICA-producing human monoclonal B-lymphocyte lines from a patient with newly diagnosed IDDM. The successful generation of monoclonal autoantibodies from lymphocytes of peripheral blood involved immortalization with Epstein–Barr virus (EBV), the selection for IgG-positive immortalized cells, and identification of IgG ICA-producing lines by indirect immunofluorescence on frozen sections of human pancreas. Although the screening methods applied were confined to the detection of ICA, the monoclonal antibodies surprisingly recognized the islet cell protein GAD as target autoantigen.

MATERIALS AND METHODS

Immortalization of B Lymphocytes and Selection for IgG-Positive Cells. Mononuclear cells were isolated from the peripheral blood of patients with IDDM showing ICA titers from negative to 4480 Juvenile Diabetes Foundation (JDF) units (see Table 1). Values above 80 JDF units were evaluated by interpolation of the standard curve obtained with sera of the ICA proficiency program (13). B lymphocytes were immortalized by infection with EBV (14) and sorted for IgG-positive cells using streptavidin-conjugated magnetic polystyrene beads (Dynabeads M-280; Dianova, Hamburg, F.R.G.) coated with a biotin labeled goat anti-human IgG antibody [Fc-specific, $F(ab)_2$ fragments, 10 μg of antibody/mg of beads; Dianova]. EBV-infected mononuclear cells and coated beads were mixed at a beads:target cell ratio of 5:1 and incubated with rolling for 30 min at 4°C. Rosetted cells were collected in the magnet and all other cells were removed. After washing three times in 140 mM NaCl/3 mM KCl/8 mM $NaHPO_4$/1.5 mM KH_2PO_4, pH 7.4 (PBS)/0.1% bovine serum albumin (BSA), cells were seeded in microtiter wells (2×10^2 to 6×10^3 cells per 100 μl per well) in Iscove's modified Dulbecco's medium with 15% fetal calf serum on a feeder layer of irradiated human peripheral blood mononuclear cells (5×10^4 cells per 100 μl per well, 40 Gy). Cells were fed once a week and cultured for 3–4 weeks until the medium of the wells acidified.

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; ICA, cytoplasmic islet cell antibody(ies); GAD, glutamate decarboxylase; MICA, monoclonal islet cell antibody(ies); BSA, bovine serum albumin; JDF, Juvenile Diabetes Foundation; EBV, Epstein–Barr virus; GABA, γ-aminobutyric acid.

Antibody Screening and Cloning. Supernatants of the EBV-transformed B lymphocytes were screened by the conventional ICA test (15). In brief, an indirect immunofluorescence test was performed on unfixed cryostat sections of human pancreas from a donor with blood group O using fluorescein isothiocyanate-conjugated goat anti-human IgG antibodies for detection. Lines producing islet cell antibodies were expanded to 0.5–1 × 10^6 cells and cloned repeatedly on a single cell level in microtiter wells with Iscove's medium/15% fetal calf serum/5% supernatant of lipopolysaccharide-activated monocytes (16) on a feeder layer of irradiated human peripheral blood mononuclear cells (5 × 10^4 per well, 40 Gy).

Determination of Heavy and Light Chains. Subclasses of IgG antibodies were determined in an ELISA. Microtiter plates were coated (1 hr, 20°C) with a sheep anti-mouse IgG (Fc-specific) antibody (10 μg/ml), and unspecific binding was blocked by 1% BSA in PBS. Mouse monoclonal subclass-specific antibodies (Boehringer Mannheim) (10 μg/ml) were incubated as capture antibodies, followed by washing with PBS/0.05% Tween 20. After incubation of culture supernatants or standards (Nordic, Bochum, F.R.G.) (100 μl per well) and washing in PBS/0.05% Tween 20, peroxidase-conjugated anti-human IgG antibody (Boehringer Mannheim) was incubated and reaction was developed after washing with 2,2'-azinodi[3-ethylbenzthiazoline sulfonate (6)] (Boehringer Mannheim) and hydrogen peroxide. All incubations were done for 1 hr at 20°C. Light chains were determined in an immunodot assay as described (17) using anti-human κ light chain- or anti-human λ light chain-specific mouse monoclonal antibodies (Dianova).

Crossreactivity with Other Human Tissues. Preformed complexes of IgG monoclonal antibodies and horseradish peroxidase-conjugated goat anti-human IgG antibodies were generated in ELISA microtiter wells as described (18), incubated for 2 hr at 4°C on cryostat sections of human tissues, and washed three times, 10 min each, in cold PBS. Binding of peroxidase-complexed monoclonal islet cell antibody (MICA) was detected by staining with aminoethylcarbazole and hydrogen peroxide.

Blocking Studies. Preformed molecular complexes of the monoclonal antibodies and horseradish peroxidase-conjugated goat anti-human IgG antibodies were generated as described above and diluted in PBS to the final dilution giving good staining of islet cells in the histochemical test. Unfixed sections of human pancreas were preincubated with culture medium, supernatants of the different MICA lines (1 hr, 20°C), or different ICA-positive sera or normal control sera (diluted 1:8 in PBS, 30 min, 20°C) and washed for 15 min in PBS. Thirty microliters of the preformed peroxidase complexes of the different MICA diluted in PBS was incubated with the pretreated sections (2 hr, 4°C) and binding was detected as described above. Sections were finally counterstained with Mayer's hemalum.

Immunoprecipitation Experiments. Isolated human islets (19) were cultured in RPMI medium/10% fetal calf serum for 1–3 days labeled with [^{35}S]methionine (specific activity, >1000 Ci/mmol; 1 mCi per 1000 islets; 1 Ci = 37 GBq), and extracts were immunoprecipitated with 0.5 ml of culture supernatant of the MICA lines, with culture medium, or with 25 μg of affinity-purified IgG1 of an unrelated human monoclonal antibody and with 100 μl of protein A-Sepharose or protein G-Sepharose (for MICA 5), respectively, as described (30). Samples were subjected to PAGE (12%) and processed for fluorography. Gels were dried and exposed to Kodak X-Omat AR films at −70°C.

Immunotrapping Assay. Culture medium, supernatant of the MICA lines and of an unrelated human monoclonal IgG1 antibody, as well as sheep anti-GAD serum were incubated with protein A-Sepharose or protein G-Sepharose (Pharmacia) for 3 hr at 4°C. Immune complexes were then incubated with 250 μl of a supernatant of rat brain or pig brain homogenate [10 min, 12,000 × g; 10% (wt/vol) in 0.2 mM pyridoxal phosphate, pH 7.2] as a source of GAD overnight at 4°C. Immunotrapped enzyme activity was detected by the amount of labeled CO_2 formed after addition of 25 mM [^{14}C]glutamatic acid (CEA, Saclay, France) as a substrate.

Table 1. Immortalization of lymphocytes

Patient	Age, years	Duration of IDDM	Serum ICA level,* JDF units	ICA-positive primary wells
1	20	4 years	—	—
2	22	11 years	—	—
3	23	11 years	5	—
4	16	1 year BD	20	1
5	19	ND	20	—
6	31	5 years	40	3
7	27	ND	80	—
8	40	ND	80	—
9	29	ND	80	1
10	28	7 months	120	2
11	27	ND	120	3
12	15	ND	160	2
13	23	2 years	180	—
14	13	ND	280	—
15	39	7 years	296	2
16	32	ND	560	7
		3 months	560	13
17	28	ND	640	—
18	28	5 months	640	1
19	17	ND	1920	1
20	21	1 month	4480	1

ND, newly diagnosed; BD, before diagnosis.
*ICA levels >80 JDF units were extrapolated from the standard curve obtained by the JDF-ICA Workshop sera.

RESULTS

Generation of Human Monoclonal Autoantibodies. The B lymphocytes originated from 19 patients with IDDM and one prediabetic individual. By selecting IgG-producing immortalized B cells, we eliminated the IgM-producing B lymphocytes, which predominate in the pool of peripheral blood lymphocytes and usually produce antibodies of low affinity and specificity (20). Screening of 5400 B-lymphocyte culture supernatants by an indirect immunofluorescence test, which is the conventional test for detection of ICA (15), identified 37 supernatants positive for IgG ICA (Table 1). Cells from all 37 ICA-positive wells were subjected to repeated cloning at the single cell level. Six IgG ICA-producing monoclonal cell lines could be stabilized at the single cell level. Due to the instability and low cloning efficiency of EBV-transformed B-cell lines (21), 31 of the primary positive cultures did not survive this procedure or stopped their antibody production during the cloning steps. The stable six monoclonal islet cell antibodies, termed MICA 1–6, originated from lymphocytes of one person (no. 16, Table 1). The serum of this patient showed the typical cytoplasmic staining pattern of ICA and no antibodies against other endocrine tissues were detectable. MICA 1–6 are of the IgG class and have been stable in cell culture now for >1 year.

Table 2. Subclass and light chain distribution of MICA 1–6

	MICA 1	MICA 2	MICA 3	MICA 4	MICA 5	MICA 6
IgG subclass	G1	G1	G1	G1	G3	G1
Light chain	λ	λ	λ	κ	κ	λ

Table 3. Blocking of MICA staining by preincubation with ICA-positive and normal human sera

Preincubation	ICA level of diluted serum, JDF units	Reaction					
		MICA 1	MICA 2	MICA 3	MICA 4	MICA 5	MICA 6
PBS/BSA	—	++	+	++	+++	+++	++
Serum 1	280	(+)	−	−	−	−	−
Serum 2	160	+	+	(+)	−	+	(+)
Serum 3	40	+	+	−	+++	+++	++
Serum 4	20	(+)	+	++	+++	+++	++
NHS 1–3	—	++	+	++	+++	+++	++

PBS/BSA, PBS with 0.1% bovine serum albumin; NHS, normal human sera. Semiquantitative evaluation of staining: +++, very strong; ++, strong; +, weak; (+), very weak; −, no staining of islet cells.

Subclass Distribution of MICA 1–6. Analysis of the IgG heavy chain subclasses of MICA 1–6 showed that MICA 1–4 and 6 were of subclass IgG1, the predominant IgG subclass of ICA in patients with IDDM (22, 23), and contained either κ or λ light chains. MICA 5 was of subclass IgG3 with a κ light chain (Table 2).

Blocking of MICA Staining by ICA-Positive Sera. Monoclonal antibodies with ICA characteristics should compete with ICA-positive sera for binding in the conventional islet cell antibody test. We tested four high-titered ICA-positive sera from patients with IDDM and three ICA-negative sera from normal individuals for their ability to inhibit the binding of preformed peroxidase complexes of MICA 1–6 to cryostat sections of human pancreas. Nonspecific blocking of MICA binding occurred when undiluted IDDM and normal sera were applied. With 1:8 diluted sera, however, a complete blocking or significant reduction of the MICA staining was observed with high-titered ICA-positive sera but not with normal human sera. Sera with low ICA titers were less potent blockers of MICA binding (Table 3).

Tissue and Species Specificity of MICA 1–6. The tissue and species specificity of the MICA was analyzed by an indirect immunofluorescence test or by immunohistochemistry using preformed MICA–peroxidase complexes (18) to avoid unspecific staining via Fc receptors and detection of endogenous immunoglobulin in tissues. All MICA showed a staining of cytoplasmic components of the islet cells, whereas exocrine pancreas cells were not stained (Fig. 1*A*). Within the islet MICA 1–6 predominantly stained β cells (data not shown). In addition to human islets, MICA 1–6 recognized islets from cow, pig, rat, and monkey. Among human tissues none of the MICA showed reactivity with thyroid, adrenal gland, pituitary, liver, stomach, or intestinal tissue. However, they all reacted with neurons in rat cerebellar cortex and stained, among other structures, somata and extending dendrites of Purkinje cells (Fig. 1*B*). Five oligoclonal IgG ICA-positive supernatants of lymphocytes from five other patients, which could not be stabilized as monoclonal antibodies, all showed a similar staining of islet cells and reacted with sections of rat cerebellar cortex. Only three of the supernatants (derived from patients 4, 10, and 11) were available in sufficient amount to be tested on several human tissues and they showed the same tissue specificity as MICA 1–6.

Epitope Studies by Blocking Experiments. Complexes between single MICA and horseradish peroxidase-conjugated goat anti-human IgG were produced (18) and used in blocking studies to analyze whether MICA 1–6 recognize different epitopes on their target molecules. Binding of each MICA was assessed after preblocking of pancreatic sections with each of the six different MICA or with culture medium. Fig. 2 presents the data for MICA 1. Binding of MICA 1 was blocked by MICA 1 or 3 but not by MICA 2, 4, or 6 or culture medium. This result suggests that MICA 1 and 3 recognize identical or closely related epitopes. A summary of the results of all blocking experiments done with the individual MICA is given in Table 4. MICA 2 and 5 seem to share the same epitope, which is distinct from the MICA 1 or 3 epitope. Furthermore, MICA 4 and 6 each recognize a different and distinct epitope, which may be sterically close to the MICA 2 or 5 epitope. These results are consistent with four distinct target epitopes being recognized by MICA 1–6 in islet cells.

Identification of the Islet Cell Target Antigen. Immunoprecipitation experiments were carried out to assess whether any of the MICA recognize protein antigens in islet cells. Isolated human islets were labeled with [^{35}S]methionine, extracted with a Triton X-100 lysis buffer, and immunoprecipitated using supernatants of the MICA-producing lines and protein A-Sepharose or protein G-Sepharose. Results from these experiments are shown in Fig. 3. All MICA precipitated a protein of 63–65 kDa from human islet extracts, whereas affinity-purified human IgG1 of an unrelated human monoclonal antibody as well as culture medium did not. A protein of the same molecular mass was precipitated from porcine islets with MICA 1–6 (data not shown).

The Target Autoantigen of MICA 1–6 Is GAD. The results shown above demonstrated that the target antigen of the MICA was expressed in islets and in cerebellar neurons and had a relative molecular mass of 63–65 kDa in pancreatic islets. A diabetes-relevant autoantigen, which is expressed in pancreatic β cells and GABAergic (GABA, γ-aminoisobutyric acid) neurons in the central nervous system, was recently identified as the enzyme GAD (7). To analyze the possible GAD activity of the target antigen of MICA 1–6 the enzymatic activity of the immunocomplexes was tested in an

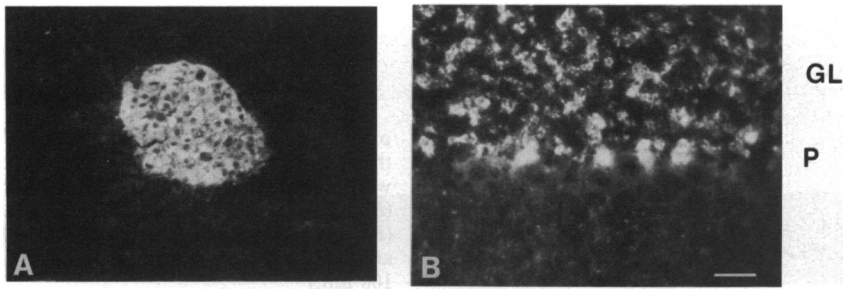

FIG. 1. Reactivity of MICA 5 with human pancreas (*A*) and rat cerebellar cortex (*B*) after indirect immunofluorescence staining with fluorescein isothiocyanate-conjugated goat-anti-human IgG antibodies. GL, granular layer; P, Purkinje cells. (Bar = 100 μm.)

Table 4. Epitope studies by blocking experiments

	Blocking of binding of MICA 1–6					
Preincubation	MICA 1	MICA 3	MICA 2	MICA 5	MICA 6	MICA 4
Medium	–	–	–	–	–	–
MICA 1	+	+	–	–	–	–
MICA 3	+	+	–	–	–	–
MICA 2	–	–	+	+	+	–
MICA 5	–	–	+	+	+	–
MICA 6	–	–	+	+	+	+
MICA 4	–	–	–	–	+	+

immunotrapping assay. The monoclonal antibodies were coupled to protein A-Sepharose or protein G-Sepharose beads and incubated with extracts of rat or pig brain, showing GAD enzyme activity. When coupled to protein A, all MICA except MICA 5 immunotrapped GAD activity from rat brain, whereas an unrelated human monoclonal IgG1 antibody and culture medium did not (Table 5). Human IgG3 does not bind to protein A. Therefore MICA 5, which is the only antibody of subclass IgG3, failed to immunotrap GAD activity from rat brain extracts using protein A. However, protein G-bound MICA 5 immunotrapped GAD activity about five times higher than control values from pig brain extracts. Compared to the other MICA this activity is still low. MICA 5 may have a lower affinity for GAD and/or protein G, as suggested by a much weaker GAD band in immunoprecipitates with MICA 5/protein G than with the other MICA. A binding of MICA 5 close to the catalytic site of GAD could also explain a lower enzymatic activity of immunotrapped GAD. However since MICA 2 recognizes an epitope, which is closely related or identical to the epitope of MICA 5 and immunotraps high GAD enzyme activity, this explanation is less likely. In sum, the data demonstrate that the target antigen recognized by all MICA is the enzyme GAD.

DISCUSSION

In this paper we describe the isolation and characterization of six human monoclonal ICA isolated from lymphocytes of a patient with IDDM. All islet-reactive human monoclonal antibodies derived from patients with IDDM so far have been of the IgM class and showed crossreactivity with various human tissues (10–12). Such polyreactive autoantibodies of the IgM class can be isolated from normal individuals as well as endocrine patients and do not seem to be disease related (20). To exclude such irrelevant IgM-producing clones, only IgG-secreting cells were selected among the immortalized lymphocytes for further culture, antibody screening, and single cell cloning. The screening procedure applied to select for islet-specific autoantibodies was restricted to the detection of ICA. This strategy resulted in a successful production of stable human monoclonal ICA of the IgG class (MICA 1–6). Besides islet cells, all MICA showed reactivity with GABAergic neurons of the cerebellar cortex but not with various other tissues. High-titered ICA-positive IDDM sera were shown to compete with the MICA for binding to islet cell sections, confirming that the monoclonal antibodies recognize common ICA epitopes. Although ICA have been suggested to recognize gangliosides (8, 9), the target antigen of the MICA was identified as an islet cell protein of 63–65 kDa. Antigen precipitated by the monoclonal antibodies from rat or pig brain homogenate revealed GAD enzyme activity. These data demonstrate that MICA 1–6 are IDDM-related

Table 5. Immunotrapping of GAD enzyme activity from brain homogenate

	Immunotrapping with protein A*		Immunotrapping with protein G†	
Antibody	$^{14}CO_2$ formed, cpm	Relative GAD activity	$^{14}CO_2$ formed, cpm	Relative GAD activity
Reference serum	4965	1.000	2700	1.000
Culture medium	81	0.016	31	0.012
Unrelated IgG1	136	0.027	43	0.016
MICA 1	5962	1.201	3308	1.225
MICA 2	4348	0.875	1660	0.615
MICA 3	3328	0.670	2802	1.037
MICA 4	3612	0.727	2062	0.764
MICA 5	108	0.022	209	0.077
MICA 6	1973	0.397	1402	0.519

*Experiment done with rat brain extracts.
†Experiment done with pig brain extracts.

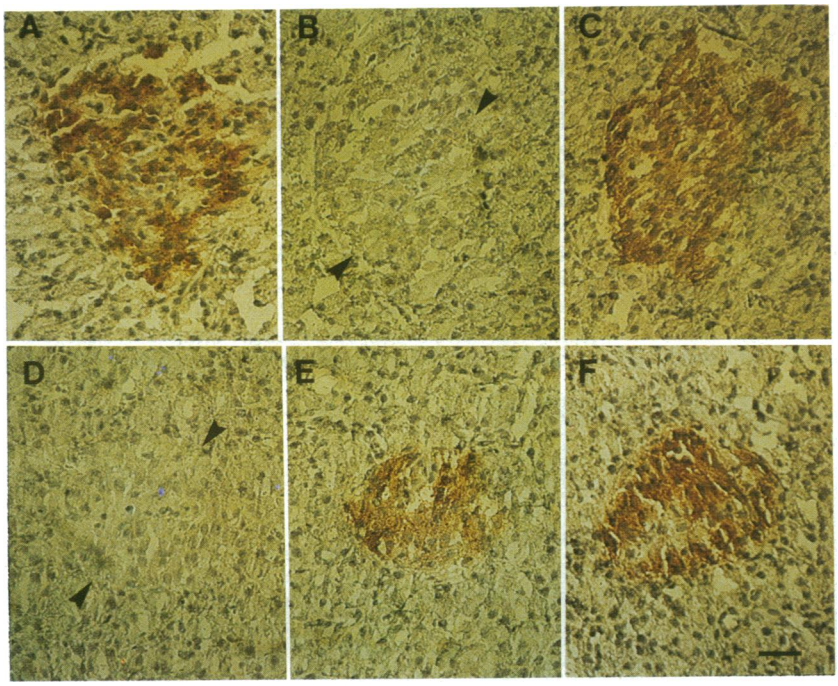

FIG. 2. Binding of the MICA 1–peroxidase complex to pancreas sections that had been preblocked by incubation with culture medium (*A*) or with MICA 1 (*B*), MICA 2 (*C*), MICA 3 (*D*), MICA 4 (*E*), and MICA 6 (*F*). Negative islets in *B* and *D* are marked by arrowheads. (Bar = 100 μm.)

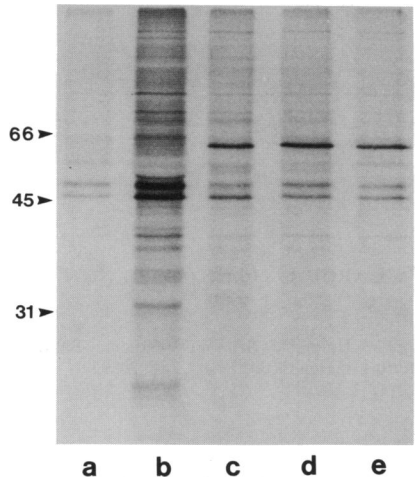

FIG. 3. Fluorogram of SDS/PAGE gel. [^{35}S]Methionine-labeled human islet cells were lysed with a Triton X-100 buffer, and extract of 500 islets per sample was immunoprecipitated with protein A-Sepharose and MICA 1 (lane c), MICA 4 (lane d), and MICA 6 (lane e), with an unrelated human monoclonal IgG antibody (lane b), and with culture medium (lane a). Molecular masses are in kDa.

human monoclonal ICA, which recognize the protein GAD. GAD, therefore, is among the target antigens of ICA.

Human GAD autoantibodies in sera from patients with IDDM were described as 64-kDa antibodies by Baekkeskov *et al.* (4). These antibodies have been shown to be present in ≈80% of newly diagnosed IDDM patients as well as individuals with early phases of β-cell destruction (24–26). The 64-kDa antibodies are of the IgG class (27). The only test to study the reactivity of these antibodies until recently was by immunoprecipitation of *in vitro* labeled islet cells followed by SDS/PAGE analysis and autoradiography. Some 64-kDa-positive sera have been reported to be negative in the indirect immunofluorescence assay for ICA (4, 25). The monoclonal human GAD antibodies developed in this study were selected by the indirect immunofluorescence assay for ICA. They could therefore be used to test the tissue specificity of human GAD antibodies by immunohistochemical methods. Immunoreactivity of MICA 1–6 in our studies was restricted to islet cells and neurons of the cerebellar cortex. This is consistent with the tissue-restricted expression of GAD activity demonstrated with a mouse monoclonal antibody to GAD (28). Anti-GAD serum from sheep was reported to show a β-cell-specific staining pattern (29), which is consistent with our observation that MICA 1–6 stained predominantly β cells within the islet.

Analysis of subclass and light chain distribution among the MICA as well as their epitope recognition confirm the polyclonal nature of the immune response to autoantigens. Although our results are so far confined to one individual, the predominance of IgG1 class among our monoclonal antibodies probably reflects the high frequency of this IgG class in ICA-positive sera (22, 23).

All of the monoclonal antibodies stabilized in the present study originated from the same individual. They strongly suggest that GAD is the major target antigen of ICA in this patient. In addition, oligoclonal ICA from five other individuals showed reactivity with islets and GABAergic neurons in the cerebellar cortex. It is therefore likely that these oligoclonal ICA were also directed to GAD, which is selectively expressed in these tissues. GAD may therefore be a common target antigen of ICA.

The monoclonal antibodies presented here are all specific for the smaller form of GAD, GAD_{65}, and do not recognize the larger form of GAD, GAD_{67} (W.R., Y. Shi, and S. Baekkeskov, unpublished results). They represent valuable tools for affinity purification of GAD_{65} from different sources. Furthermore, they will enable the identification of human autoantigenic epitopes in GAD. The characterization of autoantigenic B-cell epitopes in GAD is important for attempts to identify possible molecular mimicry with viral or bacterial epitopes, which may be involved in the initial induction of autoimmune processes resulting in IDDM.

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