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A Monoclonal Antibody Applicable for Determination of C-Peptide of Human Proinsulin by RIA

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ABSTRACT

BALB/c, (BALB/c x B10.A)F_1 and (BALB/c x B10)F_1 hybrid mice were immunized with C-peptide of human proinsulin. The (BALB/c x B10.A)F_1 hybrids were the best responders and yielded 3 hybridomas secreting specific monoclonal antibodies. One of them, C-PEP-01, bound the C-peptide with high affinity (K_{as} = 1.1 x 10^9 l/mol), cross-reacted fully with human proinsulin but not with insulin, glucagon or somatostatin and apparently recognized the regions of C-peptide comprising amino acid residues 8-13 and 25-31. A RIA system could be set up employing this monoclonal antibody suitable for estimation of C-peptide concentrations in a diagnostically useful range (1-50 ng/ml).

INTRODUCTION

Immunologically human insulin is practically indistinguishable from the animal insulins used for treatment of diabetics. This is due to a very high degree of structural homology: human insulin differs from porcine and bovine insulin in just one and three amino acid residues, respectively. That is why it is not possible to use anti-insulin antibodies for determination of residual insulin secretion in diabetics. Instead, it is necessary to use the estimation of C-peptide of human proinsulin for this purpose. The C-peptide is a part of proinsulin synthesized in the beta-cells of pancreatic Langerhans cells. Still before secretion from the Langerhans cells, most of the proinsulin molecules (86 amino acid residues) are cleaved into two cystine-linked chains of insulin (51 amino acids), C-peptide (31 amino acids) and two dipeptides. Insulin and the C-peptide are secreted in equimolar ratio together with residual proinsulin - approximately 2-6% (1).

Radioimmunological or enzymoimmunological estimation of the C-peptide concentrations in sera and urine is currently based on the use of polyclonal, usually guinea pig, antibodies. The polyclonal antibodies usually do not have sufficiently high specificity which causes problems especially in the case of estimation in urine. This drawback can be in principle solved by the use of appropriate monoclonal instead of polyclonal antibodies. Preparation of relatively low-affinity anti-C-peptide monoclonal antibodies has been described by Madsen *et al.* (2, 3) but these cannot be used (due to the low affinity) for routine estimation of the C-peptide in human body fluids. Similarly, the anti-C-peptide monoclonal antibodies commercially available from Novo (Denmark) are recommended only for use in immunohistochemistry.

In the present work we studied in some detail the immunogenicity of human C-peptide in mice and prepared high-affinity monoclonal antibodies suitable for estimation of the C-peptide concentrations in body fluids.

MATERIALS AND METHODS

The modified human C-peptide containing N-terminal tyrosine residue allowing introduction of the radioactive label and an internal marker for monitoring the binding to carrier (norvaline) was prepared as described by Bendlová *et al.* (4) and used for immunization and for radioimmunoassay (RIA). The ^{125}I-C-peptide was prepared with the Iodo-gen method by K. Mudra (ÚVVVR, Prague). For immunization, the 1-Tyr-C-peptide was coupled to bovine thyreoglobulin or serum albumin (BSA) by means of glutardialdehyde (5). On the average, seven molecules of C-peptide were found to be coupled to one molecule of BSA and about 40 C-peptide molecules to one molecule of thyreoglobulin. This antigen was emulsified in Bacto Adjuvant, complete or incomplete Freund, Difco Laboratories (Detroit, MI, USA). Polyclonal antibody used was the guinea pig antiserum against C-peptide developed with the same immunogen as that used for MAb preparation (K_{as} = 6.3 x 10^{10} l/mol).

Animals

Female mice of the following strains and F_1 hybrids (bred and maintained in the animal colony of the Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague) were used: BALB/c (BALB/c x B10.A)F_1, (BALB/c x B10)F_1. The nu/nu mice of the BALB/c background were provided by Dr. M. Holub (Institute for Clinical and Experimental Medicine, Prague).

Hybridoma preparation

Fusion with the Sp2/0-Ag14 plasmacytoma cell line (6) (obtained from Dr. A. Hedin, Wenner-Gren Institute, Stockholm, Sweden), hybridoma cloning and production of ascitic fluid were performed using modifications of standard techniques (7). Briefly, spleen cells (5×10^7, washed in RPMI 1640) from the immunized mice were fused with the Sp2/0-Ag14 cells (2×10^7) by means of 50% polyethylene glycol (PEG) 1500 (cell culture tested: Sigma, St. Louis, MO, USA). After washing with HAT medium, the fused cells were distributed into the wells of ten 96-well trays containing a layer of mouse peritoneal feeder cells in HAT medium. In all fusions, hybridoma colonies grew on the average in 70% of the wells. From the 7th day onwards, supernatants of the wells containing growing hybridoma colonies were tested for the presence of anti-C-peptide antibodies by RIA (see below). Ascitic fluids were produced in peritonea of mice sensitized with Pristane (Sigma). The isotypes of the MAbs were determined by an ELISA procedure (8) and isoelectric points by isoelectric focusing in horizontal 5% polyacrylamide gel slab. The approximate concentrations of the MAbs in the ascites were estimated by polyacrylamide gel electrophoresis in carbonate buffer (9).

Precipitation RIA

The sera of the immunized mice and the hybridoma supernatants were assayed for the presence of anti-C-peptide antibodies by a liquid phase precipitation RIA, as described elsewhere (10).

Determination of avidity and specificity of the MAbs

Avidity and specificity of the MAbs were tested by RIA. ^{125}I-Tyr-C-peptide was prepared

by means of the iodo-gen procedure (Pierce, Rockford, IL, USA) and purified by HPLC. All reactants were diluted in a solution containing 0.15 M NaCl, 20 mM sodium phosphate pH 7.4 and 0.1% BSA. Reaction mixture (100 µl diluted antibodies, 100 µl tracer solution, 100 µl diluted standards or substances tested for cross-reactivity) was incubated overnight at 4 °C and the antibody-bound radioactivity was precipitated by addition of 100 µl of 1% human IgG and 400 µl of 20% PEG. Association constants were determined from Scatchard plots, cross-reactivities were calculated from molar concentrations of the compounds in 50% intercept of the RIA system. Fragments of human C-peptide molecule were synthesized using the Fmoc- and Boc-solid phase procedure (4).

The following peptides were tested:

X-Glu^{1}-Ala-Glu-Asp-Leu-Gln-Val-Gly^{8}-Gln-Val-Glu-Leu-Gly^{13}-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala^{25}-Leu-Glu-Gly-Ser-Leu-Gln^{31}-*Y*

I, *X*=H, *Y*=OH; II, *X*=H-Tyr, *Y*=Gly-OH; III, *X*=H-Tyr, *Y*=Ape-OH

H-Tyr-Gly^{8}-Gln-Val-Glu-Leu-Gly^{13}-Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala^{25}-Leu-Glu-Gly-Ser-Leu-Gln^{31}-*X*-OH

IV, *X*=Gly; V, *X*=Ape; VI, H-Tyr-Gly^{8}-Gln-Val-Glu-Leu-Gly^{13}-Gly-OH
VII, H-Tyr-Ala^{25}-Leu-Glu-Gly-Ser-Leu-Gln^{31}-Gly-OH
VIII, H-Ahx-Gln-Val-Gly^{8}-Gln-Val-Glu-Ile-Gly^{13}-Gly-NH_2

Cross-reactivity of human, porcine and bovine insulin, glucagon (all Novo, Copenhagen, Denmark), somatostatin (Stilamin, Serono, Freiburg, FRG) and human proinsulin (NIH, Bethesda, MD, USA) was tested up to the concentration of 100 ng/ml.

RESULTS AND DISCUSSION

Immunogenicity of the Human C-peptide in Mice

The most efficient among several immunization procedures tested was the scheme shown in Table 1. The C-peptide coupled to bovine thyreoglobulin given in the first doses was less

TABLE 1. Protocol for Immunization of Mice with Human C-peptide

Dose	*Day of application*	*Route of injection*	*The antigen form*[a]
1	0	s.c.; 50 ng/0.2 ml; belly	complete adjuvant[b]
2	21	s.c.; 50 ng/0.2 ml; footpads	incomplete adjuvant[b]
3	35[c]	s.c.; 50 ng/0.2 ml; belly	complete adjuvant[b]
4-5	50 and 51	i.v.; 30 ng/0.2 ml; tail vein	in saline
6-7	52 and 53	i.p.; 30 ng/0.2 ml	in saline

[a]C-peptide coupled to bovine thyreoglobulin was used in doses 1, 2 and 3, conjugate with BSA in doses 4-7
[b]Bacto adjuvant incomplete (complete) Freund
[c]In some animals the immunization continued every 21 days as in dose 3

immunogenic than the conjugate with BSA (data not shown). Ten days after the second dose, the blood was taken from the tail vein and tested for the presence of anti C-peptide antibodies by RIA. The mice responding poorly to the C-peptide did not increase significantly the specific antibody titer even after further five doses of the antigen. Therefore, we continued the immunization only in those mice the sera of which diluted 1:100 exhibited in RIA a signal at least 10-fold higher than the nonspecific background (negative control).

Immunogenicity of the C-peptide in Homozygotes and Heterozygotes .

C-peptide coupled to BSA is immunogenic in BALB/c mice. The (BALB/c x B10 A)F_1 hybrids were better responders than BALB/c while the (BALB/c x B10)F_1 hybrids responded poorly to this antigen (Table 2).

TABLE 2. Immune Response to C-peptide of the BALB/c and F_1 Hybrid Mice

Strain	*Total number of animals*	*Number of high-(intermediate-) responders* [a]	*Number of fusions* [b]	*Hybridomas obtained*
BALB/c	18	5	4	none
(BALB/c x B10.A)F_1	10	6	3 [c]	C-PEP-01, -02, -03
(BALB/c x B10)F_1	5	0	0	none

[a] As judged by RIA; see text.
[b] The fusions were made with spleen cells of the best responders.
[c] C-PEP-01 was obtained from one fusion, C-PEP-02 and C-PEP-03 from two other fusions in which a mixture of two spleens was used.

Of 7 fusions performed, three hybridomas, named C-PEP-01, C-PEP-02 and C-PEP-03, were obtained that produced C-peptide specific monoclonal antibodies. All these hybridomas originated from the fusions using the spleen cells of immunized (BALB/c x B10.A)F_1 hybrids. The clones were stable as ascertained by cloning repeated three times. These results demonstrate that immune responsiveness of mice to human C-peptide is under genetic control; the A^k or E^k alleles are obviously associated with good responsiveness. While the MHC control of immune response to insulins of various species in mice was studied in detail (11), no similar studies have as yet been performed with C-peptide.

Adaptation of the Hybridomas Originating from the (BALB/c x B10.A)F_1 Spleen Cells to Growth in BALB/c Mice

In our experience, most of the hybridomas obtained by fusion of the spleen cells of the (BALB/c x B10.A)F_1 hybrids with the Sp2/0 plasmacytoma cell line can successfully grow in the peritoneum of the BALB/c mice in spite of the relatively strong histoincompatibility. In agreement with this observation, the C-PEP-02 and C-PEP-03 hybridomas grew in the BALB/c peritoneum and produced the MAbs into the ascitic fluid.

However, the C-PEP-01 hybridoma, which produced the MAb with most desirable properties, grew only when very high inoculum (10^7 cells) was used in the (BALB/c x B10.A)F_1

hybrids but not at all in the BALB/c mice. This unfavorable feature could be reversed by growing the C-PEP-01 cells (inoculum of 5×10^6) transiently in the peritoneum of nu/nu mice. When 10^7 of these cells which had passed through the nu/nu mice were injected into the BALB/c peritoneum, they grew well in 3 of 6 cases. The hybridoma adapted in this way then grew well in 100% of cases in BALB/c mice even when inocula as small as 5×10^5 of cells were used. This behavior of the C-PEP-01 hybridoma reflects the known phenomenon of tumor adaptation to new genetic environment and is in agreement with the idea of tumor progression and clonal evolution (12).

Characterization and Specificity of the MAbs

The characteristics of the three MAbs against the C-peptide are shown in Table 3. The association constant of the C-peptide-MAb C-PEP-01 complex was $K_{as} = 1.1 \times 10^9$ l/mol. The useful working range of the RIA system based on this antibody was 1-50 ng/ml (0.3-16 nmol/l) of the C-peptide. No cross-reactivity was found with human, porcine and bovine insulins, glucagon and somatostatin. On the other hand, human proinsulin cross-reacted almost fully with this MAb. Small differences between the affinity of C-peptide and proinsulin apparent from Fig. 1a are probably due to the conformational constraints of the connecting peptide in the proinsulin molecule (3). The cross-reactivities of fragments of the C-peptide are shown in Fig. 1b and in Table 4. Only the fragments containing the sequences AA_{8-13} and AA_{25-31} (corresponding to the sequences 40-45 and 57-63 of human proinsulin) reacted strongly with the C-PEP-01 MAb. Changes in the marginal parts of the C-peptide molecule had no effect on the interaction. Thus, the MAb reacts with the central part of the C-peptide molecule, probably with the hydrophilic segments 8-13 and 25-31. Similar results were also obtained in a previous study on immunogenicity of proinsulin (3).

TABLE 3. Properties of the MAbs

MAb	*Isotype*	p*I*	*Typical concentration of MAb in the ascitic fluid* (mg/ml)	K_{as} (l/mol)
C-PEP-01	IgG1	6.0 - 6.7	10 - 12	1.1×10^9
C-PEP-02	IgG1	6.5 - 6.8	2 - 4	10^7
C-PEP-03	IgG1	6.8 - 7.2	3 - 5	10^7

The monoclonal antibody C-PEP-01 is suitable for RIA determination of human C-peptide in serum or plasma of Type 2 diabetics and controls. In the case of Type 1 diabetics with long duration of disease, it appeared insufficient. The main field of applicability of our monoclonal antibody in human diagnostics should be the determination of C-peptide in the urine. As shown in Fig. 2, there is a good correlation between the results of C-peptide estimation in urine by polyclonal and monoclonal RIA, but the absolute values obtained by the latter method were significantly lower ($p < 0.01$ in paired Student's t-test). This can be explained by the presence of a broad spectrum of peptide fragments present in the urine that may cross-react with the polyclonal antiserum. Therefore, the results obtained by means of a highly specific MAb system are probably more realistic.

The differences are statistically significant (paired Student's t-test, $p < 0.01$).

It can be concluded that the C-PEP-01 MAb is fully acceptable for diagnostic radioimmunological estimation of the C-peptide concentrations in urine samples of diabetic patients. The low-affinity antibodies C-PEP-02 and C-PEP-03 could possibly be useful for immunoaffinity purification of proinsulin.

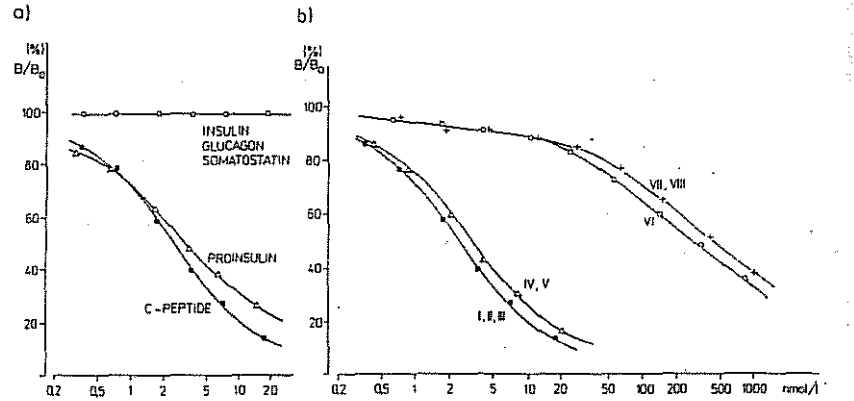

FIGURE 1. Cross-reactivity of the C-PEP-01 MAb with Hormones (a) and C-peptide Fragments (b). The structures of the C-peptide fragments are shown in Materials and Methods. The ratios of bound/unbound ^{125}I-C-peptide are plotted *vs.* the concentrations of the substance tested in the RIA system.

TABLE 4. Specificity of the C-PEP-01 MAb in the RIA System Using ^{125}I-Tyr-C-peptide

Peptide	*Cross-reactivity* [a]
Human C-peptide (I)	100
Insulin human	< 0.001
porcine	< 0.001
bovine	< 0.001
Glucagon	< 0.001
Somatostatin	< 0.002
Proinsulin (human)	87
Modified C-peptides [b]	
II	98
III	98
C-peptide fragments [b]	
IV	82
V	79
VI	0.8
VII	0.6
VIII	0.6

[a] 50% intercept calculated from molar ratios.
[b] Structures see Materials and Methods.

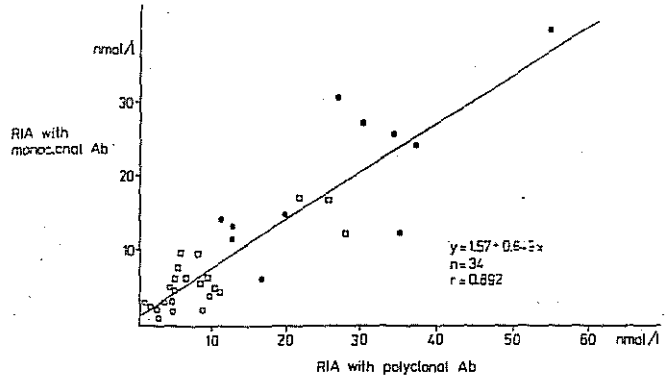

FIGURE 2. Correlation between Polyclonal and Monoclonal RIA Systems for C-peptide in Urine. Conditions (i.e., standards, tracer, buffers, separation procedure etc.) were identical in both systems. The values obtained using monoclonal antibody C-PEP-01 were significantly lower (paired Student's t-test, $p < 0.01$). Controls ⊏, Type 1 diabetics o, Type 2 diabetics (newly diagnosed) ●.

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