Characterization of Seven C-peptide Antisera

O. K. Faber, M.D., C. Binder, M.D., J. Markussen, Ph.D., L. G. Heding, Ph.D., V. K. Naithani, Ph.D., H. Kuzuya, M.D., P. Blix, B.S., D. L. Horwitz, M.D., and A. H. Rubenstein, M.B., F.R.C.P., F.A.C.P., F.C.P.(S.A.), Klampenborg and Bagsvaerd, Aachen, and Chicago

SUMMARY

The plasma C-peptide immunoreactivity (CPR) in 10 normal subjects varied considerably when measured with different antisera in parallel assays. The CPR level correlated with the blank "CPR" value measured in plasma devoid of C-peptide and to a lesser degree with the sensitivity of the standard curves obtained with the individual antisera.

Storage of plasma samples at different temperatures and for different lengths of time before the analyses were carried out resulted in further variation in the CPR results. This was caused by a time- and temperature-dependent fall in CPR, which was more pronounced with some antisera than with others. This sensitivity to storage of plasma did not correlate with the antigenic characteristics of the antisera as determined by their reactivity with 11 specific fragments of the C-peptide molecule.

The contribution of human proinsulin to the CPR concentration in normal subjects was considered to be negligible even though the relative immunoreactivity of human proinsulin and C-peptide ranged from 11 to 143 per cent among these antisera.

These results suggest that differences in C-peptide antisera are a major reason for the variation in the concentration of circulating CPR as measured in different C-peptide immunoassays. DIABETES *27* **(Suppl.1):170-77, 1978.**

The wide range of serum C-peptide concentrations reported in normal fasting subjects, namely, from 0.29 to 0.82 pmol per milliliter,[23,33,99,171,209,243] suggests there are important differences in the assays employed. Previous studies have shown that the level of serum C-peptide immunoreactivity (CPR) depends on the antiserum used.[171,240] Heding[171] suggested that the nonspecific displacement of tracer was the property of an antiserum that was mainly responsible for the variation in reported CPR levels. As no systematic evaluation of the commonly available antisera has been carried out, we have characterized seven C-peptide antisera according to their method of production, cross-reactivity with human proinsulin, sensitivity, and reactivity with plasma devoid of C-peptide and with various fragments of C-peptide. Furthermore, the importance of the assay tracer was determined by measuring the binding characteristics of two tyrosylated synthetic C-peptide preparations of different origin. Finally, as the serum C-peptide concentration has been reported to decrease during storage,[240] the effects of storage at different temperatures and for different lengths of time were also examined.

From Hvidøre Hospital (OKF and CB), Klampenborg, Denmark, the Laboratory of Peptide Chemistry (JM), Novo Industry A/S, and the Novo Research Institute (LGH), Bagsvaerd, Denmark, the Deutsches Wollforschungsinstitut an der Technischen Hochschule (VKN), Aachen, West Germany, and the Department of Medicine (KH, PB, DLH, and AHR), University of Chicago, Chicago, Illinois.

Address reprint requests to Ole K. Faber, Hvidøre Hospital, Emiliekildevej 7, DK-2930 Klampenborg, Denmark.

MATERIAL AND METHODS

Antisera

Five of the antisera examined were raised in guinea pigs: M1181 by immunizing the animals with crude b-component obtained by gel filtration of first crystals of human insulin,[171] antiserum G by injecting a mixture of human proinsulin and its intermediate forms

separated from a crude extract of human insulin,[240] and M1219, M1227, and M1230 by immunizing the animals with synthetic benzyloxycarbonyl human C-peptide coupled to human albumin.[99] Antiserum Y was produced in rabbits immunized with a synthetic connecting segment of human proinsulin (kindly provided by Dr. N. Yanaihara, Shizouka, Japan), while antiserum C was raised by a similar technique (kindly provided by Calbiochem, La Jolla, Cal.).

Table 1 contains a summary of the methods used to produce the antisera.

Standards

Synthetic human C-peptide[327] was used as the standard except in studies concerned with characterization of the two iodinated tracers. In these particular studies natural human C-peptide was used.[343]

The fragments 1-7, 8-31, 18-31, 23-31, and 25-31 were obtained from the corresponding protected human C-peptide intermediates,[327] while the fragments 5-13, 14-22, and 14-24 were prepared for the present studies. The details of their syntheses, purification, and characterization will be published elsewhere.

The fragment 1-24 was obtained from postmortem human pancreas by extraction and purification as described by Markussen et al.[289]

Fragments 4-31 and 4-28 were synthesized by Dr. N. Yanaihara according to previously described methods.[510,513] The concentrations of all the fragments in their final dilution were measured by amino acid analysis.

Human proinsulin was isolated from a crude preparation of crystalline human insulin.[240]

Tracers

Synthetic tyrosyl-C-peptide iodinated with ^{125}I was used as the tracer in most of the studies.[328]

For the studies of the influence of different tracers, synthetic tyrosylated human connecting segment prepared by Yanaihara et al.[513] (A) and synthetic tyrosyl human C-peptide prepared by Naithani et al.[328] (B) were iodinated by the method of Hunter and Greenwood[191] as modified by Freychet et al.[124] Initially 4 μg. of each preparation was dissolved in 0.01 ml. 0.3 M phosphate buffer, pH 7.4, and stored at $-20°$ C. until used. At the time of iodination, 0.04 ml. 0.3 M phosphate buffer, pH 7.4, was added to each tube to give a final volume of 4 μg. synthetic human C-peptide per 0.05 ml. phosphate buffer; 4 mCi. ^{125}I was then added to each preparation. A 0.33-mg./ml. solution of chloramine T was prepared immediately before use and 0.02 ml. was added to each iodination mixture. After three minutes of gentle agitation, 0.005 ml. of a solution of sodium metabisulfite (0.66 mg./ml.) was added to stop the reaction. After the addition of 0.1 ml. of assay buffer, the iodination mixtures were gel-filtered on 0.8×10 cm. columns of BioGel P-30, equilibrated in the assay buffer as previously described.[240] About 60 fractions (0.3 ml. per tube) were collected and counted.

Radioimmunoassays

The immunologic reactivities of the synthetic human C-peptide standard, the fragments, and human proinsulin in the concentration range 0 to 1.0 pmol per milliliter were determined by using the seven different antisera in the assay system previously described.[99,171] At a suitable dilution (table 1), 100 μl. of antiserum was incubated with 100 μl. of standard, fragment, proinsulin, or unknown sample. Twenty hours later, 100 μl. ^{125}I-tyr-C-peptide (0.04 pmol) was added, and after a further 24 hours of incubation, the bound tracer was precipitated with ethanol and counted.

The iodinated tracers were studied with a modification of the Morgan and Lazarow[317] double-antibody immunoassay as described by Kuzuya et al.[240] The assay buffer was a 0.133 M borate buffer, pH 8.0, containing 0.5 per cent bovine serum albumin.

Antigenic characteristics of the antisera

Each antiserum was characterized by measuring its reactivity with 11 specific fragments of the C-peptide molecule. From these results it was possible to define the particular amino acid sequence to which the antibodies in each antiserum were predominantly directed.

Sensitivity

The sensitivity of the standard curves obtained with the different antisera was expressed as the per cent decrease of bound tracer, when 0.10 pmol per milliliter C-peptide was added.

Nonspecific displacement of tracer

The nonspecific displacement of the tracer was determined with plasma devoid of C-peptide (0-plasma). The plasma was from 10 insulin-dependent diabetic patients treated with insulin for more than 10 years who did not show an increase in the plasma C-peptide concentration after intravenous administration of 1 mg. glucagon. The C-peptide concentration in such a plasma sample was considered to represent the nonspecific displacement of tracer or blank value of the particular antiserum.

Proinsulin reactivity

The relative immunoreactivity of proinsulin and

C-peptide was calculated from the C-peptide concentration that gave the same displacement of tracer as 1.0 pmol per milliliter proinsulin. The result was expressed in per cent. The magnitude of the cross-reactivity of proinsulin in the lower working range of the C-peptide assay was expressed as the concentration of proinsulin giving the same displacement of tracer as 0.05 pmol per milliliter C-peptide.

Binding studies of iodinated tracers

Varying dilutions of antisera Y (1:2,500 to 1:20,000) and M1230 (1:5,000 to 1:50,000) were incubated with the ^{125}I-synthetic tyrosyl human C-peptide preparations (A and B) (20,000 c.p.m. per 0.1 ml.). The first antibody reaction was carried out at 4° C. for 24 hours, and the final volume in each assay tube was 1.0 ml.

In the second antibody reaction (24 hours at 4° C.), the antigen-antibody complex was precipitated in the Y system with 0.1 ml. of normal rabbit serum (1:80) and 0.1 ml. goat antirabbit globulin serum (1:30). In the M1230 system, 0.1 ml. normal guinea-pig serum (1:300) and 0.1 ml. rabbit antiserum raised against whole guinea-pig serum (1:18) were used.

Antibody-bound labeled peptide was separated from free tracer by centrifugation at 4° C. for 20 minutes; the supernatants were then decanted and precipitates counted.

Fasting plasma C-peptide immunoreactivity

C-peptide immunoreactivity (CPR) was determined in plasma from 10 fasting nondiabetic subjects. The blood samples were collected in plastic tubes containing Trasylol (Bayer, 450 KIE per milliliter blood) and heparin (Novo, 45 I.U. per milliliter blood). After centrifugation, the plasma was pipetted into plastic tubes and stored at −25, −10, 4, and 22° C. CPR in freshly drawn plasma and in plasma stored for 1, 4, 12, and 24 weeks was measured in parallel assays with six of the antisera.

RESULTS

Characterization of antisera

The characteristics of the seven antisera are summarized in table 1. Irrespective of the antigen used, antisera with greater sensitivity also showed low, nonspecific displacement with the C-peptide–free plasma samples.

The cross-reactivity of proinsulin varied from 11 to 143 per cent of that of C-peptide on a molar basis. The lowest reactivity of proinsulin was found with antisera raised against synthetic C-peptide (M1219 and M1230), while a higher reactivity was found with the antisera raised against the connecting segment (C and Y). As expected, the greatest degree of cross-reactivity was found with the proinsulin antisera (G and M1181).

Characterization of the antisera according to their reaction with fragments of C-peptide indicated that the small fragments 1-7, 5-13, 14-22, and 25-31 did not compete with the tracer for antibody-binding sites. Antiserum M1227 reacted with fragment 14-24 on an equimolar basis with C-peptide. It was the only antiserum that reacted with this fragment. Fragments 1-24, 4-28, 4-31, and 8-31 reacted with all the antisera, whereas only antisera M1230 and M1219 reacted with the carboxy-terminal fragments 18-31 and 23-31. The reactivities of the antisera with proinsulin and with the major fragments of C-peptide are shown in figure 1.

TABLE 1

Characterization of seven antisera used in human C-peptide immunoassays. 0-plasma = plasma devoid of C-peptide. PI = human proinsulin. CP = human C-peptide. Z-CP = Benzyloxycarbonyl-C-peptide.

Antiserum	M1181	M1219	M1230	G	Y	C	M1227
Produced by	Heding[171]	Faber et al.[98]	Faber et al.[99]	Kuzuya et al.[240]	Yanaihara[240]	Calbiochem	Faber et al.[99]
Antigen	Crude PI	Z-CP	Z-CP	Crude PI	Connecting segment	Connecting segment	Z-CP
Coupling	0	Human albumin	Human albumin	0	0	0	Human albumin
Animal	Guinea pig	Guinea pig	Guinea pig	Guinea pig	Rabbit	Rabbit	Guinea pig
Final dilution (× 1,000)	1.8	22.5	45	7.5	15	15	30
Sensitivity (Δ tracer bound, %)	10.8	15.2	15.5	3.5	4.7	5.1	3.1
0-plasma (pmol/ml.)	0.02	0.02	0.03	0.4	0.09	0.11	0.10
Mean and Range	0.00-0.07	0.00-0.05	0.00-0.06	0.01-0.08	—	0.03-0.22	0.00-0.22
Reactivity of PI (%)	100	27	11	143	62	52	47
PI corresponding to CP = 0.05 (pmol/ml.)	0.13	0.18	0.30	0.11	0.11	0.13	0.15
Binding site	8-17	C-terminal	C-terminal	8-17	4-31	4-31	14-24

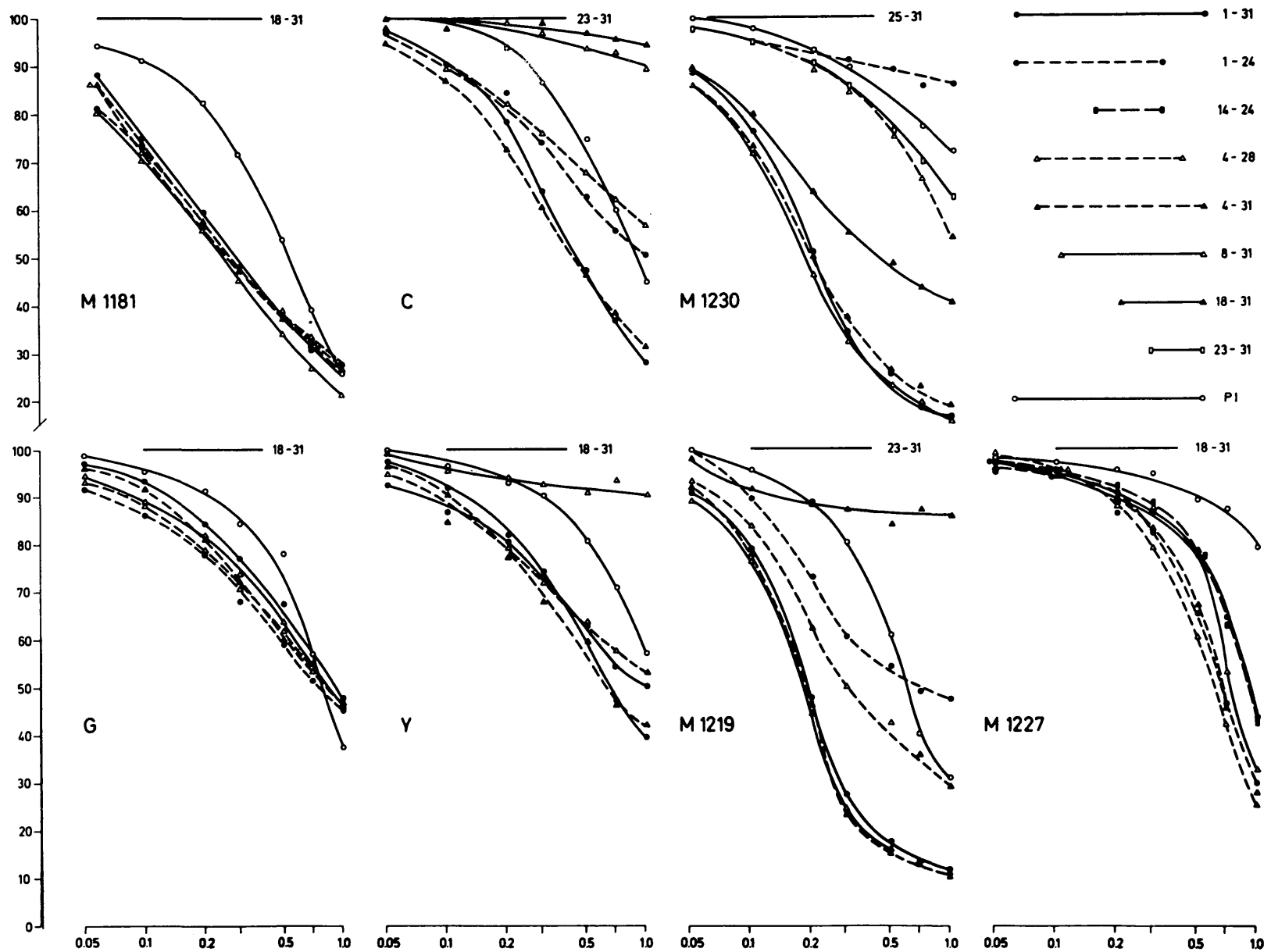

FIG. 1. Standard curves of human proinsulin, C-peptide, and fragments of C-peptide determined with seven antisera.

The two antisera M1181 and G, raised against different crude human proinsulin preparations, had similar patterns of reactivity with the various fragments. Equimolar reactivity was found with the fragments 1-24, 4-28, 4-31, and 8-31. No reactivity was found with the fragments 1-7 and 18-31. These results suggest that these antisera are predominantly directed against the sequence 8-17.

The two antisera, Y and C, raised against the connecting segment also showed similar patterns of reactivity. They reacted on an equimolar basis with C-peptide and the fragment 4-31, whereas fragment 8-31 showed little reactivity. This shows that the amino acid sequence 4-7 is of major importance in determining the reaction between the C-peptide and these antisera. However, as the reaction with fragment 1-24 was not equimolar, this finding suggests that other regions of the C-peptide molecule may have a role, albeit to a lesser degree. For instance, the lower reactivity of fragment 4-28 as compared to fragment 4-31 indicates that the three carboxy-terminal amino acids also are involved in the reactivity of these antisera.

Two of the C-peptide antisera, M1219 and M1230, showed similar characteristics. They reacted on an equimolar basis with the major carboxy-terminal fragments, 4-31 and 8-31, and had little reactivity with the amino-terminal fragment 1-24. The binding site for these antisera, therefore, seems to be directed towards the carboxy-terminal part of the C-peptide molecule. This concept is supported by their low reactivity with fragment 4-28 and their significant reactions with the relatively short carboxy-terminal fragments 18-31 and, especially, 23-31.

Antiserum M1227, which was obtained with the same immunization procedure as M1230, differed markedly in its immunologic characteristics. The fragments 1-24, 4-28, 4-31, and 8-31 reacted equally or even better than the C-peptide standard, and fragment 14-24 reacted on an equimolar basis. The two amino acids, 23 and 24, seem to be of particular importance for this antiserum, because no cross-reactivity was shown with fragment 14-22.

Characterization of tracers

The elution patterns of the two ^{125}I-synthetic human tyrosyl C-peptide preparations are shown in figure 2. Tracer A (specific activity 546 mCi./mg.) eluted in one symmetric peak that was clearly separated from the second, free-iodine peak. This elution pattern and specific activity was consistent with previous iodinations. In contrast, tracer B eluted in two overlapping peaks. These two peaks will be referred to as labeled peaks B-I and B-II. Peak B-II was smaller and less discrete than peak B-I. The specific activity of tracer B (peaks I and II) was 574.3 mCi./mg.

Points circled in figure 2 denote tubes of labeled material used in the binding studies. Figure 3 illustrates the per cent binding of the tracers A, B-I, and B-II with varying dilutions of antisera Y and M1230. The conditions and dilutions in each of the binding assays were similar except for the labeled preparations used.

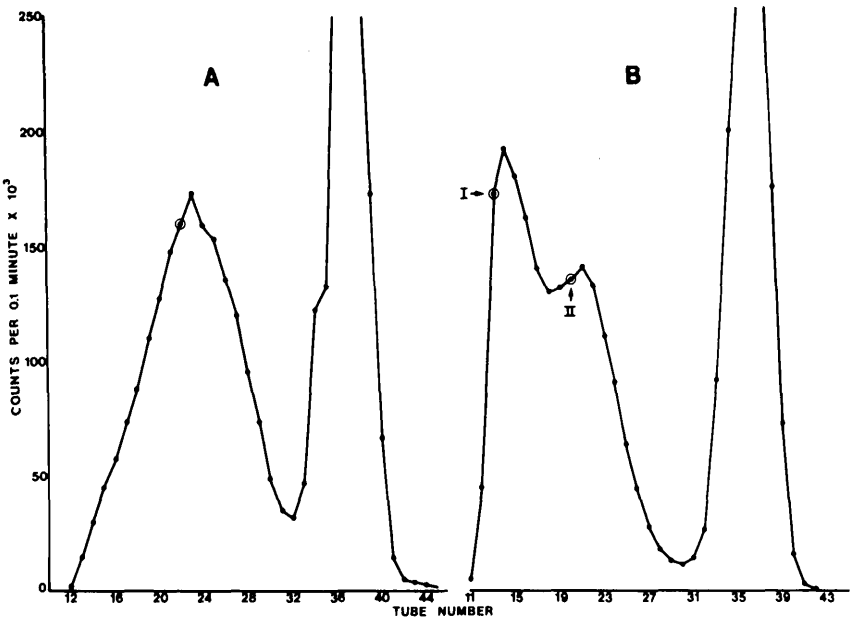

FIGURE 2

Gel filtration pattern of ^{125}I-synthetic human tryosyl C-peptide preparations A and B (see text) on BioGel P-30 eluted with 0.133 M borate buffer. Points circled denote tubes of labeled material used in the binding studies.

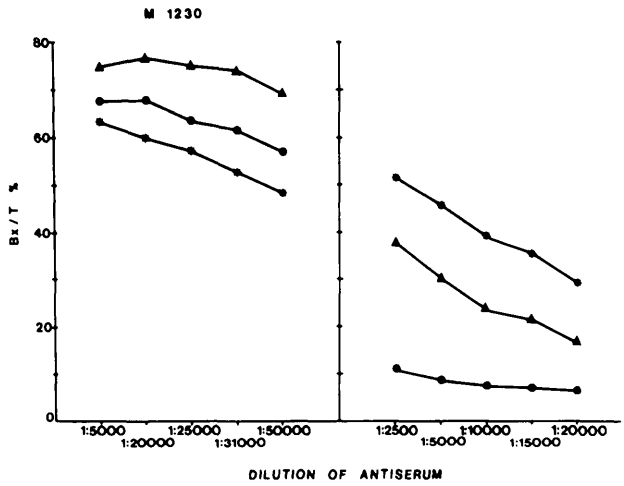

FIG. 3. Binding of ^{125}I-synthetic human tyrosyl C-peptide Preparations A (stars), B-I (circles), and B-II (triangles) with antisera M1230 and Y at different dilutions.

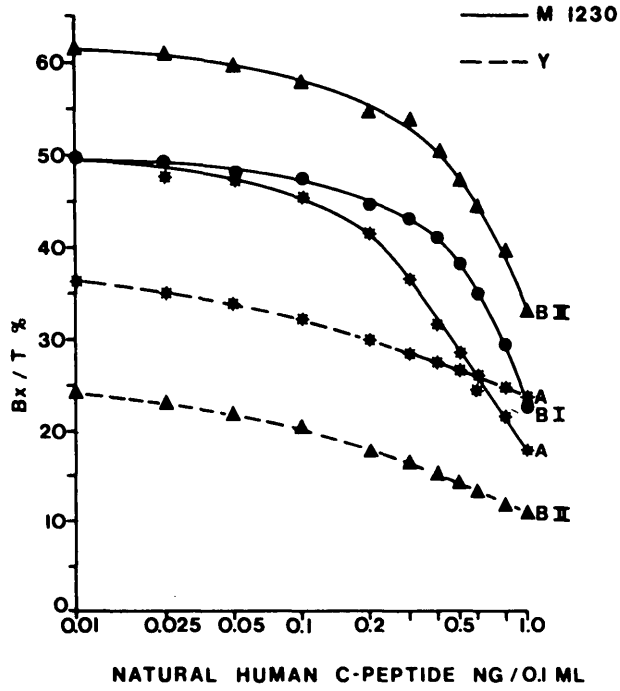

FIG. 4. Standard curves constructed with antisera M1230 and Y by use of natural human C-peptide as standard and ^{125}I-synthetic human tyrosyl C-peptide preparations A (stars), B-I (circles), and B-II (triangles) as tracers.

In the M1230 system, ^{125}I-B (peak II) material was bound to the greatest extent. The ^{125}I-A preparation was bound to the smallest extent. In the Y system, the ^{125}I-A tracer showed the greatest degree of binding, while ^{125}I-B (peak II) reacted less well. The ^{125}I-B tracer (peak I) was only bound to a small degree (< 11 per cent).

Figure 4 compares standard curves obtained with antisera Y (final dilution 1:15,000) and M1230 (final dilution 1:25,000) using the two labeled preparations A and B-I. The standard curve with B-II was measured with M1230 only. A logit-log transformation of the curves showed that the two standard curves in the Y system were not parallel, the one using label B-II exhibiting a steeper slope. In contrast, all three standard curves appeared parallel in the M1230 system, with the labeled preparation A being displaced to the greatest extent.

CPR in plasma

CPR was measured in fresh Trasylol plasma taken from 10 normal, fasting subjects. The values obtained with six of the antisera varied appreciably (table 2). The highest values were found with the antisera M1227, C, and G. These antisera also gave the highest nonspecific displacement. The CPR concentrations

TABLE 2

C-peptide immunoreactivity (CPR) in Trasylol-heparin-plasma from 10 normal fasting subjects. Average and range of CPR concentrations (pmol/ml. × 10^{-2}) determined with six antisera after different lengths of storage at different temperatures are shown.

Temperature (°C.)	Fresh	22		4			−10				−25			
Duration (wk.)		1	4	1	4	12	1	4	12	24	1	4	12	24
M1181	36	31	19	35	36	24	30	32	33	32	36	35	32	34
	28-50	22-47	14-31	29-51	26-56	16-40	24-44	22-50	20-53	21-52	27-49	24-55	26-51	27-57
M1219	41	29	21	35	31	28	38	33	29	27	32	35	31	37
	30-58	24-35	17-30	29-49	27-42	23-41	28-57	25-48	24-42	24-30	28-48	28-57	28-45	29-51
M1227	65	57	46	62	59	54	64	61	60	55	61	61	59	61
	50-89	43-78	32-69	50-86	47-80	40-79	48-87	50-80	48-83	40-79	48-76	48-84	46-88	45-90
M1230	36	26	15	33	30	26	34	33	33	27	35	37	33	38
	26-59	19-45	11-28	26-57	21-43	19-39	28-50	24-52	21-52	19-39	26-53	27-56	27-49	28-53
G	54	50	32	52	46	38	55	50	43	39	49	47	37	39
	42-64	35-68	19-46	35-64	26-63	27-57	46-68	28-62	27-58	29-54	38-64	29-66	26-61	20-68
C	56	46	34	62	53	40	55	53	47	45	55	58	52	54
	41-74	37-65	25-55	47-86	41-72	32-61	42-77	41-71	31-66	34-65	43-69	44-76	41-74	41-72

decreased with time, most pronouncedly in plasma kept at room temperature (22° C.) and measured with antisera G or M1219. In plasma stored at −10° C., a significant decrease in CPR was generally found after four weeks. After 24 weeks, CPR values decreased further. The smallest average decline in CPR values was 11 per cent with antiserum M1181.

In samples collected in Trasylol and stored at −25° C. for 24 weeks, no significant decrease in CPR levels was measured with antisera M1181, M1230, and C. The average decrease in CPR measured with antisera M1219, M1227, and G was 10, 6, and 28 per cent, respectively.

DISCUSSION

The considerable variation in fasting CPR values in normal subjects determined in different C-peptide assays[1-6] was also observed when CPR was measured with six antisera in parallel assays in the present study. The results suggest that differences in the antisera are the main reason for the variation reported in the normal fasting CPR level.

The highest CPR values were found with antisera that gave a high, nonspecific displacement of the tracer. The variation in this parameter, however, could not quantitatively account for the total variation in fasting CPR values.

Considerable variation in the nonspecific displacement was observed between samples taken from different subjects. There was no correlation between the nonspecific displacement measured with the different antisera in the individual "0-plasma" samples. Analysis of a considerable number of "0-plasma" samples consequently is necessary for establishing the average nonspecific displacement for a given antiserum.

The nonspecific displacement did not correlate with the titer of the antiserum. On the other hand, the nonspecific displacement of each individual antiserum decreased with increasing degrees of dilution.

The lowest mean values for fasting CPR were obtained with the antisera having the highest sensitivity. To some extent, this correlation can be explained by the lower nonspecific displacement measured with these antisera.

Plasma CPR has at least two components, C-peptide and proinsulin. Varying reactivity of proinsulin with the different antisera, therefore, might contribute to the variation in the average fasting CPR in normal plasma. The concentration of proinsulin in fasting normal subjects, however, is too low to contribute significantly to the CPR concentration. Thus, a contribution of 0.05 pmol per milliliter to the plasma CPR requires a proinsulin concentration that is 12 to 33 times higher than its normal fasting concentration of 0.009 pmol per milliliter.[175] Such high proinsulin concentrations may, however, be seen in insulin-treated patients who have residual β-cell function, because proinsulin which circulates bound to insulin antibodies in these patients, is eliminated from the circulation slowly.[117]

C-peptide–related peptides (other than proinsulin) have been considered to play a role in contributing to the heterogeneity of serum CPR.[240] It is uncertain, however, whether differing reactivity of antisera to CPR fragments contributes significantly to the variation in fasting CPR concentrations. Thus, the mean value for fasting CPR measured with antiserum G was 50 per cent higher than that measured with antiserum M1181, despite the fact that both antisera were directed towards the sequence 8-17 of human C-peptide.

The storage experiment confirmed that CPR in plasma declines considerably with time. The degree of decline depended on the storage temperature and on the antiserum used. The storage conditions, therefore, may influence the results appreciably.

It is clear that the properties of the individual antiserum influence the measured levels of CPR considerably. However, studies with the two tracers suggest that the iodinated peptide used in the C-peptide radioimmunoassay is also an important variable to be considered when evaluating this assay method. The two synthetic tyrosyl human C-peptide preparations had similar specific activities but different elution patterns when iodinated by a similar technique. With the two antisera Y and M1230, differences in the binding of each label were apparent and the relative reactivities of the tracers varied in the two systems (figure 3).

Differences in competition of the three tracers with the natural human C-peptide standard for antibody-binding sites were also noted. In the Y system, a logit-log plot of the standard curves indicated that the labeled peptide B-II was displaced more effectively than the A preparation by the natural human C-peptide standard. Moreover, the standard curves run in this system were not parallel. In contrast, standard curves of antiserum M1230 with the three tracers were parallel, but the labeled peptide A was displaced to a greater extent at each concentration of the standard.

Preliminary results did indicate differences in the

CPR values of serum samples assayed by the different label preparations but the same antiserum and natural human C-peptide standard. However, more extensive dilution studies need to be done to establish the validity of these findings.

Other factors, alone or in combination, may also be of importance in influencing serum CPR values. Thus the fasting CPR in normals measured with antiserum G in the present study was 0.54 pmol per milliliter, but the same antiserum gave a value of 0.30 pmol per milliliter when a different assay technique, tracer, and standard were used.[240] Furthermore, in the latter assay system, the serum CPR remained constant for as long as seven months of storage.

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