Abnormal Relaxin Secretion during Pregnancy in Women with Type 1 Diabetes[1]

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To test the hypothesis that relaxin may play a role in the fetal abnormalities associated with pregnancy in type 1 diabetic women, we previously compared gestational relaxin concentrations in diabetic and clinically normal women using a porcine relaxin radioimmunoassay (RIA): Serum immunoactive relaxin was significantly ($P < 0.001$) elevated in the diabetic women. To confirm and extend this work in a larger group of subjects, we have now used an enzyme-linked immunosorbent assay (ELISA) specific for human H2 relaxin (the normal human gene product) to determine immunoactive serum relaxin concentrations in serial samples from 61 Type 1 diabetic and 21 normal pregnant women. Samples from 22 of the diabetic and nine of the normal women were also directly compared in the porcine relaxin RIA. ELISA-determined serum relaxin was higher ($P < 0.001$) at 24 and 36 weeks of pregnancy in type 1 diabetic women than in controls, confirming previous findings. However, the geometric mean increase in immunoactive relaxin concentration in identical samples from pregnant diabetic women over that of controls was significantly greater with the RIA than with the ELISA (271% vs 44%; $P < 0.001$). To investigate this discrepancy, the specificity and epitope selectivity of the RIA and the ELISA were compared using several synthetic polypeptides, including human relaxins H1 and H2, and relaxin and insulin derivatives. Both assays showed great specificity, but the porcine RIA selectively identified the epitopes of the receptor-binding domain of the relaxin B chain and cross-reacted strongly with H1 and H2 relaxins. In contrast, only the H2 peptide was detected by the ELISA antiserum. Therefore, the marked discrepancy between the RIA and the ELISA could be due to the presence in the diabetic samples of another relaxin-like molecule in addition to the normal H2 relaxin. The biological consequences of elevated serum relaxin in diabetic pregnancy remain to be elucidated. Exp Biol Med 228:33–40, 2003

Key words: H1 relaxin; H2 relaxin; type 1 diabetes; human pregnancy

Relaxin is a 6-kD polypeptide hormone, structurally related to insulin and the insulin-like growth factors (see Ref. 1 for review). In women, relaxin is produced by the corpus luteum, both in the luteal phase of the menstrual cycle and throughout pregnancy, whereas in men, relaxin is secreted by the prostate gland and appears in seminal plasma (1). In human beings, there are at least three genes, H1, H2, and H3, coding for relaxin (1–3). The H1 and H2 gene products have similar conformational features but differ by 13 amino acids in their sequences (2). The H3 gene product bears little homology with H1 and H2, except for the receptor binding domain (RBD) between the arginines on the B chain and the A chain glycine needed for proper conformation (3). Only the H2 gene product is expressed in the corpus luteum and is found in the circulation in women (1, 2). Both H1 and H2 genes are reportedly expressed in decidua and trophoblast (4), but *in situ* hybridization techniques were not capable of identifying the gene product(s) immunolocalized in the decidua and placenta (5). H1 and H2 are reportedly expressed in the prostate (4), but only the H2 relaxin protein has actually been found in the prostate and seminal plasma (1). H3 is expressed primarily in the brain (3).

Relaxin concentrations in human blood and tissues have been determined by several different immunoassay methods (see Ref. 1 for review). The first definitive studies on relaxin in human pregnancy were performed using rabbit antiporcine relaxin antiserum R6 in a homologous porcine radioimmunoassay (RIA; 6–8). Subsequently, homologous human relaxin RIAs were developed (9, 10), and homologous human relaxin enzyme-linked immunosorbent assays (ELISAs) have since been reported (11, 12). All of these assays have yielded similar relaxin secretory patterns in studies of human pregnancy; serum relaxin concentrations are highest in the first trimester and decrease thereafter until the time of parturition.

We previously reported that serum relaxin levels in type 1 diabetic pregnant women were markedly elevated when compared with those of normal pregnant women (13). In that study, the samples were evaluated using

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the previously mentioned homologous porcine relaxin RIA, which uses a rabbit polyclonal anti-porcine relaxin antiserum, R6. R6 has been shown to cross-react specifically with the RBD of the B chain of all relaxins thus far studied (2). When the human relaxin ELISA of Lucas *et al.* (11) became available, we were afforded the opportunity to confirm our previous results on the diabetic serum samples using an immunoassay method specific for H2 relaxin.

Accordingly, in the present study, we have measured relaxin concentrations using human ELISA to confirm and extend the observations made previously by including three times as many diabetic subjects and twice as many controls. In addition, many of the samples were also tested in parallel using the R6 porcine relaxin RIA, which had been used in the previous study (13). Although both assays revealed elevated serum relaxin in the diabetic subjects, the geometric mean difference between the normal and diabetic serum relaxin concentrations was more than 6-fold greater ($P < 0.001$) when the R6 RIA rather than the human ELISA was used on the same samples. In an effort to find an explanation for this unexpected divergence, the cross-reactions of the ELISA with human H1 and H2 relaxins, and several synthetic relaxin and insulin derivatives were compared with those of the porcine relaxin RIA. By doing so, we hoped to better characterize the utility, specificity, and epitope recognition of each of these immunoassays. No data of this type are currently available for the homologous human relaxin RIAs (9, 10), or for the more recently described human relaxin ELISA (12).

It would seem important when studying relaxin in individuals with diseases such as type 1 diabetes to be alert to the possibility that the immunoassay may actually be measuring relaxin-like substances other than, or in addition to, the presumed H2 hormone.

Material and Methods

Subjects. Subjects were recruited from the antenatal clinics and joint obstetrics/medical clinic at the Royal Victoria Infirmary (Newcastle-upon-Tyne, UK). All recruits gave informed consent, and the project had ethical approval from the Combined Ethics Committee of the Newcastle Regional Health Authority and Newcastle University. This institutional body corresponds to the IRB committees here in the United States, and is consistent with the Declaration of Helsinki of the World Medical Association.

Sixty-one women with Type 1 diabetes (IDDM) of more than 1 year's duration were studied. All diabetic subjects ovulated spontaneously and all had singleton pregnancies. Clinical details are given in Table I. The women had all been receiving insulin for at least 1 year prior to becoming pregnant. None had any medical problem other than diabetes. White classification placed 22 subjects in class B, 30 in class C, and 9 in class D. Routine management included daily home blood glucose monitoring measured by BM stick (Boehringer, Lewes, UK) and glycosylated hemoglobin determined by gel electrophoresis. Our range for normal pregnancy is 5.8%–7.3%. Subjects were divided into 47 with good control and 14 with poor control, the latter based on more than one mean daily whole blood glucose (average of four daily readings) over 10 m*M* or glycosylated hemoglobin over 10% on at least one occasion at monthly routine obstetric visits. Fifty-four women had a live healthy singleton outcome. Of these, 43 women delivered at or after 37 weeks gestation and none before 35 weeks. Birth weights were standardized for gestation and infant sex by subtracting the median expected birth weight for the relevant gestation and were on average 461 g (SD 585) greater than the median.

Seven type 1 diabetic women had bad infant outcome. Two had early fetal loss. One mother had fulminating pre-

Table I. Clinical Details of Subjects

Subjects	Weeks of gestation		
	12 [mean (± SD)]	24 [mean (± SD)]	36 [mean (± SD)]
Normal Body Weight (kg)		67.4 (8.7)	
n		21	
IDDM[a] Mgluc (m*M*/1)[b]	5.64 (1.65)	6.30 (1.88)	5.78 (1.60)
Good control			
HbA1%	7.72 (1.05)	7.09 (1.04)	7.52 (1.26)
Insulin dose (U/day)	46.3 (17.1)	53.6 (18.3)	70.5 (21.9)
Body Weight (kg)		66.4 (8.4)	
n	37	46	45
IDDM[a] Mgluc (mM/l)[b]	6.35 (1.66)	7.10 (1.35)	5.66 (2.10)
Poor control			
HbA1%	8.92 (1.48)	8.14 (1.47)	7.91 (1.56)
Insulin dose (U/day)	46.3 (11.7)	60.9 (20.6)	78.4 (22.9)
Body Weight (kg)		69.3 (9.3)	
n	8	14	14

[a] IDDM, type 1 diabetic women.
[b] Mgluc, mean of four daily glucose measurements.
n = number of subjects.

eclampsia at 27 weeks and was delivered of a normal infant (781 g) who died at 11 days. The other mother had twins, one of which died at 22 weeks, the other normal twin surviving to term. Three women suffered late intrauterine deaths at 36 weeks. No cause for these deaths was discovered; biophysical profiles 3 days prior to delivery scored 10. Autopsies showed nothing of note. Two women delivered infants with fetal anomaly, one with cleft palate, the other with gross cardiac and gut anomalies. Both infants survived.

Control subjects were 21 healthy nondiabetic women, similar (but not rigidly matched) in age, ethnicity, body size, and obstetric history to the type 1 diabetic women. The control women ovulated spontaneously, had uncomplicated pregnancies, and gave birth to single, live, healthy infants at or after 37 weeks gestation. Birth weights adjusted for gestation were 56 g (SD 297) less than the median.

Serial postprandial serum samples (venous blood) were obtained from diabetic and control women at 12 weeks (range 7–16), 24 weeks (range 21–28), and 36 weeks (range 31–38) of gestation and were stored at −70°C. Assays for insulin-like growth factor-1 (IGF1), human chorionic gonadotropin (hCG), human placental lactogen (hPL), estradiol-17β, and progesterone previously conducted on these samples have been reported elsewhere (14, 15). The relaxin data included in this report were derived from two separate collections as follows:

Collected	Number and Type	Assay	When Assayed
1987–1989	9 normal, 22 diabetic	Porcine relaxin RIA	1989
	same samples	Human relaxin ELISA	1992
1990–1992	12 normal, 39 diabetic	Human relaxin ELISA	1992–1993
Totals	21 normal, 61 diabetic	Human relaxin ELISA	1992–1993

The first collection was used for the comparison of the two immunoassay methods reported here: the homologous porcine relaxin RIA and the homologous human relaxin ELISA, described below. The ELISA data from the two collections were then combined to provide a final comparison of normal and diabetic pregnancy relaxin levels after statistical validation of this procedure.

Human H2 Relaxin ELISA. The method was used exactly as published (11). The reagents were kindly provided by Dr. Richard Vandlen (Genentech, South San Francisco, CA). Briefly, affinity-purified goat antibodies to H2 relaxin were adsorbed onto 96-well microtiter plates (Maxisorb; Nunc, Naperville, IL) overnight, washed, blocked with bovine serum albumin in phosphate-buffered saline, and then washed again. H2 relaxin standards (78–1280 pg/ml) and unknown serum samples were prepared. Nonimmune goat immunoglobulin (Ig) G (20 μg/ml) was added to all samples prior to their being pipetted into the microplate wells in duplicate (100 μl). After a 15-hr incubation at 4°C, the plates were washed, horseradish peroxidase-conjugated rabbit anti-H2 relaxin was added to each well, and the plates were incubated for an additional 4 hr at room temperature. After washing, the orthophenylene diamine substrate was added to each well and was incubated for 10 min, following which, the reaction was stopped by addition of sulfuric acid. The resulting absorbance was read at 490 nm, and the results were calculated automatically on a 450 Microplate Reader (Bio-Rad Laboratories, Hercules, CA) and presented as nanograms per milliliter H2 relaxin. Coefficients of variation ranged from 5% to 15%, and the minimal detectable concentration was 7.8 pg/tube.

R6 Porcine Relaxin RIA. The R6 homologous porcine relaxin RIA was conducted as previously described (13, 16). Purified porcine relaxin was used as standard and ^{125}I-labeled tyrosyl relaxin was used as radioligand. Antiserum R6 was used at a concentration of 1:20,000. All standards (31–2000 pg/tube) and unknown samples were run in triplicate. As has been reported previously (6), dilution curves of human pregnancy sera and porcine relaxin standards were parallel. Concentrations of relaxin in human serum were calculated as nanograms per milliliter porcine relaxin equivalents. Coefficients of variation ranged from 8% to 13%, and the minimal detectable concentration varied from 31 to 125 pg/tube, depending on the individual assay.

When synthetic human relaxin became available, concentration response curves were run comparing displacement of binding of ^{125}I-porcine relaxin to antiserum R6 by the porcine and human relaxin standards (Fig. 1). The curves were linear and parallel, as one would have expected from the previous studies. In additional experiments, porcine and human relaxin were used respectively as radiolabeled tracer, or standard, and their binding to rabbit anti-porcine relaxin antiserum R6 was investigated. The observed relaxin concentration of pooled, first trimester normal human pregnancy serum served as endpoint (Table II). Although the measured serum relaxin concentrations were similar in every combination, ranging from 1.5 to 2.7

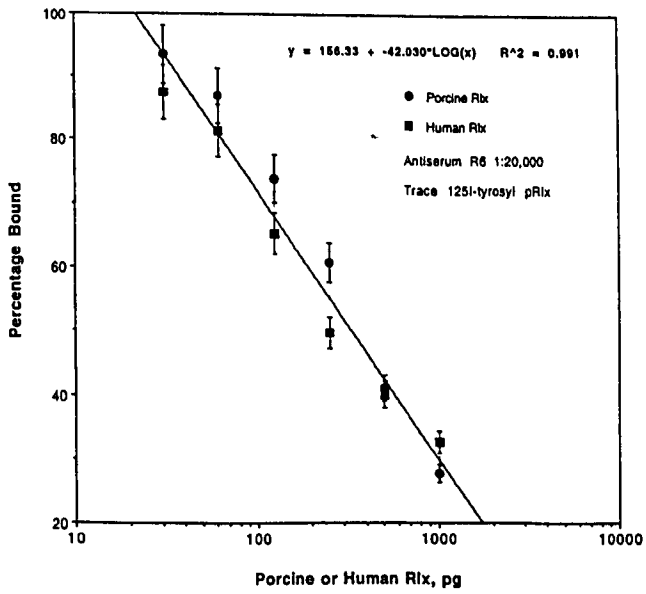

Figure 1. Comparison of porcine (pRlx) and human (hRlx) relaxin in the R6 radioimmunoassay. Each point represents the mean and SD of three assays in which each concentration was tested in triplicate.

Table II. R6 Porcine Relaxin RIA of Human Pregnancy Serum Pool against Human and Porcine Relaxin Standards and Radioligands

Assay no.	Standard (P or H)[a]	^{125}I-trace[a]	Sensitivity (pg/tube)	B50 (pg/tube)	Serum Rlx (ng/ml)
1	P	P	62	293	1.84
2	H	P	62	338	2.72
3	P	H	31	239	1.54
4	H	H	31	281	2.01

Note. In each assay, the relaxin standards (porcine = Cma[1]; human = Genentech 5788-17-1) were run in triplicate in doubling concentrations from 31–2000 pg/tube. The human pregnancy serum pool was made from 12 first trimester samples obtained from normal women. The serum was assayed in triplicate, 100 µl per tube. The porcine trace (radioligand) contained an added tyrosine.
[a] P = porcine, H = human.

ng/ml, they were higher when the human standard was used (2.01 and 2.72 ng of H2 relaxin per milliliter vs 1.54 and 1.84 ng of porcine relaxin equivalents per milliliter; Table II). In additional experiments, pooled first trimester normal human pregnancy serum samples were included as positive controls when the R6 porcine relaxin RIA was conducted. The observed concentrations were 1.09 ± 0.5ng/ml when the porcine standard was used (n = 12 assays) and 2.4 ± 0.4 ng/ml versus the human standard when it was included (n = 3 assays).

Thus, the apparent concentration of relaxin in human serum was consistently lower in the porcine relaxin RIA when measured against a porcine relaxin reference standard rather than a human relaxin standard. This finding may also account for the apparent differences in relaxin concentrations of normal pregnancy serum when assayed respectively in the porcine relaxin RIA and the H2 relaxin ELISA.

Comparison of the RIA and ELISA. In view of the foregoing, it was not considered appropriate to directly compare the results of the two assay methods because different reference standards and labeled ligands were used in each (i.e., porcine relaxin standards and ^{125}I-labeled, tyrosylated porcine relaxin in the RIA, and H2 relaxin standards and peroxidase-labeled H2 relaxin as trace in the ELISA). Thus, the units are not comparable (porcine relaxin equivalents versus human H2 relaxin). However, within each assay method, we have compared the observed relaxin concentrations found in the same serum samples from normal and type 2 diabetic women.

Characterization of the cross-reactivity of the H2 ELISA and R6 porcine relaxin RIA using synthetic relaxin and insulin peptides and derivatives. Previous work has shown that the antiserum R6 is primarily directed to epitopes of the RBD of the relaxin B-chain, especially the arginine residues at positions B13 and B17 (2). The Genentech antiserum (R9817AX) with which we were provided was reported not to cross-react with other human peptide hormones (e.g., insulin, IGF-1, hCG, follicle-stimulating hormone, luteinizing hormone, or prolactin; 11), but no information was available regarding its antigenic determinants on the human relaxin molecule. Accordingly, a battery of relaxin and insulin derivatives were tested by each method to determine the relative selectivity and specificity of the RIA and the ELISA. The synthesis and biological activities of these molecules (which are listed in Table III) are described elsewhere (11, 17), and only the results obtained with the two immunoassays in question will be presented here.

Statistical Analysis. The statistical methods used were analysis of variance (ANOVA) and Student or two-

Table III. Cross-Reactions of Human Relaxins, Insulin, and Their Derivatives in the R6 Porcine Relaxin Radioimmunoassay and the Human H2 Relaxin ELISA

Number	Polypeptide	R6 Porcine RIA		H2 Human ELISA		Receptor
		Amount assayed mass in pg	Amount measured pg (pRlx Std)	Amount assayed mass in pg	Amount measured pg (hRlx)	Binding assay (from Ref. 2)
1	Human Rlx 2 (hRlx2)	500	457	100	103	+
2	Human Rlx 1 (hRlx1)	500	418	100	<8[a]	+
3	Cit B13,CitB17 hRlx2	440	<62[a]	88	56	–
4	Cit B17 hRlx2	940	<62[a]	188	>125	–
5	Cit B13 hRlx2	500	<62[a]	100	51	–
6	AlaB17 hRlx2	500	<62[a]	100	75	–
7	Insulin (porcine)[b]	5000	<62[a]	100	<8[a]	–
8	GRER insulin[a]	500	447	100	<8[a]	+

[a] If any
[b] The insulin used was monocomponent porcine insulin.
[c] GRER Ins, synthetic human insulin in which the receptor binding domain of relaxin has been substituted on the B chain; Arg(B9), Glu(B10), Arg(B13), and a Gly has been substituted for Ile on A10 to correct the conformation of the A loop to that of relaxin (see Schwabe and Bullesbach, 1994 for rationale behind these substitutions).

sample t tests (on log-transformed data), as appropriate. Linear correlation was assessed (as with the other tests) using the SPSS/PC software (SPSS, Inc., Chicago, IL). Multiple comparisons were controlled using the Student-Newman-Keuls method (ANOVA) or the Bonferroni adjustment (t tests). Ninety-five percent CI refers to 95% confidence intervals. It should be emphasized that the absolute serum concentrations of relaxin obtained with the RIA (nanograms per milliliter porcine relaxin equivalents) are not directly comparable with those obtained with the ELISA (nanograms per milliliter human H2 relaxin) because different standards and ligands were used in each assay. Thus, the comparisons made were between control and diabetic subjects for the ELISA and the same for the RIA. The data are presented as the geometric means and standard deviations.

Results

Relaxin Concentrations in Normal Type 1 Diabetic Pregnancy. *Human H2 relaxin ELISA.* Changes in serum relaxin concentration (determined by ELISA) during human pregnancy in 21 normal and 61 type 1 diabetic women are shown in Figure 2, and the geometric means are given in Table IV. Serum relaxin levels at 24 weeks were significantly lower ($P < 0.001$) than at 12 weeks for both normal and type 1 diabetic women, but did not change significantly between 24 and 36 weeks of gestation. When comparing normal versus all type 1 diabetic women, relaxin levels were significantly elevated in the latter group at 24 and 36 weeks ($P < 0.001$). Log transformation and nonparametric testing achieved the same result. Consecutive values within subjects were correlated ($P < 0.001$) such that women with high relaxin levels in early pregnancy were most likely to continue to have high relaxin values through to the end of pregnancy.

Analysis of serum relaxin concentrations within subgroups of type 1 diabetic women failed to provide clues to possible consequences of elevated serum relaxin. Thus, relaxin levels in 14 patients with poor control did not differ significantly from those of patients with good control. Although nine diabetic women had a bad outcome, their problems were so varied (i.e., early fetal loss, preeclampsia, late intrauterine deaths, and fetal anomalies) that statistical treatment of the data was not considered meaningful. Serum relaxin concentrations showed no significant correlation with mean daily blood glucose, HbA1, daily insulin dose, IGF1, hPL, hCG, estradiol-17β, or progesterone, nor head and trunk size on sonar in the type 1 diabetic women (data not shown). Furthermore, analysis of combined data of all subjects showed no correlation of serum relaxin levels with maternal weight, infant or placental weight, or infant sex (data not shown).

R6 porcine relaxin RIA. The relaxin data obtained with the RIA on 62 serum samples from 22 pregnant diabetic women and 27 samples from nine normal pregnant women were very similar to those previously reported (13).

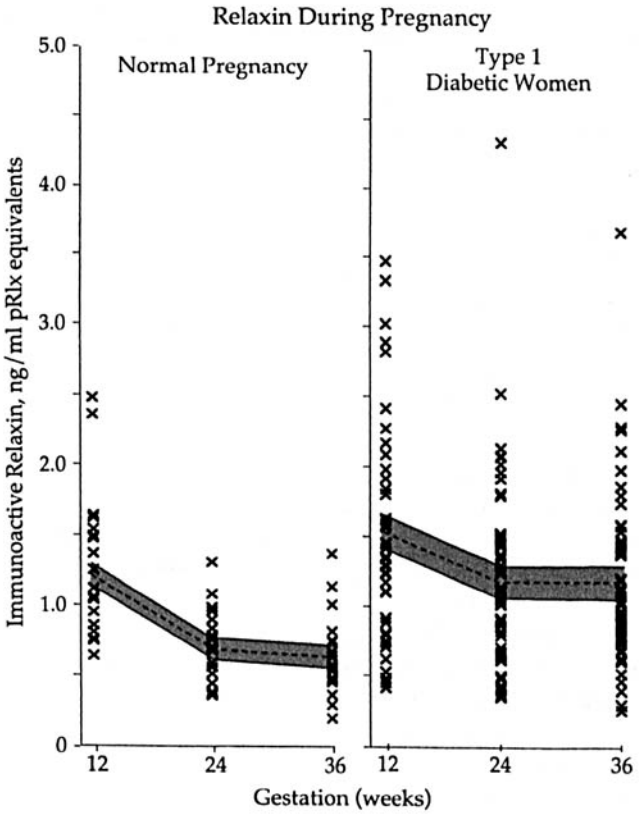

Figure 2. Relaxin concentrations in serum samples from normal and type 1 diabetic women throughout pregnancy determined by specific H2 human relaxin ELISA. Individual data points are shown as "x's," and the mean and SE's are shown as dotted lines ± shaded areas. Serum relaxin levels at 24 weeks were significantly lower than at 12 weeks in normal and diabetic subjects ($P < 0.001$), but did not change significantly between 24 and 36 weeks of gestation. Relaxin concentrations in diabetic subjects were significantly higher than those of normal subjects at 24 and 36 weeks ($P < 0.001$).

Serum relaxin concentrations (with 95% confidence limits) at 12, 24, and 36 weeks in diabetic women were 1.68 (1.44–1.96), 1.26 (1.06–1.49), and 1.35 (1.15–1.59), and in normal women were 0.49 (0.37–0.64), 0.35 (0.28–0.43), and 0.36 (0.27–0.48).

Comparison of Porcine RIA and Human H2 ELISA. Eighteen samples obtained in the second and third trimesters of pregnancy from nine normal women and 44 samples from 22 tyPEA diabetic women were assayed by both the R6 porcine relaxin RIA and the homologous H2 relaxin ELISA. When the RIA was used, the geometric mean relaxin concentration of the samples from diabetic women was 1.30 ng/ml (log value 0.26 SD 0.37), whereas that of the normal women was 0.35 ng/ml (log value1.04 SD 0.32). The difference between the normal and diabetic women (0.95 ng/ml) was highly significant ($P < 0.001$). When the same samples were tested in the human H2 relaxin ELISA, the geometric mean of the diabetic pregnant women was 0.88 ng/ml (log value –0.13 SD 0.45), whereas that of the normal samples was 0.61 ng/ml (log value –0.49 SD 0.46). The difference between the normal and diabetic women (0.27 ng/ml) was also significant ($P < 0.01$). How-

Table IV. Serum Immunoactive H2 Relaxin during Human Pregnancy

Subjects	12 weeks			24 weeks			36 weeks		
	Mean	95% CI	*n*	Mean	95% CI	*n*	Mean	95% CI	*n*
Normal	1.05	0.97–1.14	21	0.82[a]	0.77–0.89	21	0.79	0.72–0.86	21
IDDM	1.13	1.06–1.21	45	1.01[ab]	0.95–1.07	60	1.01[ab]	0.95–1.07	59
Type 1 diabetic patients with									
Good control	1.10	1.02–1.18	37	0.98	0.91–1.06	46	1.00	0.94–1.07	45
Poor control	1.28	1.07–1.54	8	1.10	0.97–1.24	14	1.04	0.89–1.21	14
Good outcome	1.12	1.04–1.21	41	1.01	0.94–1.08	53	1.01	0.95–1.07	53
Bad outcome	1.22	1.04–1.45	4	1.01	0.87–1.18	7	1.04	0.75–1.43	6

Note. Relaxin concentrations are geometric means (ng/ml) and 95% CI of the mean, measured by ELISA.
[a] Significantly different from early pregnancy: $P < 0.001$.
[b] Significantly different from normal pregnancy: $P < 0.001$.

ever, the geometric mean increase in relaxin concentration between the control and diabetic women was greater when the RIA was used than when the ELISA was used (271% vs 44%; significant interaction between diabetes and assay type, $P < 0.001$).

Cross-Reactivity of the H2 ELISA and R6 Porcine Relaxin RIA Using Synthetic Relaxin and Insulin Peptides. The data are presented in Table III. Each immunoassay was highly specific for human H2 relaxin (peptide number 1), and did not recognize insulin (peptide number 7). However, the R6 porcine relaxin RIA also reacted strongly with human H1 relaxin, whereas the human H2 ELISA did not recognize the H1 gene product (see peptide number 2 in Table III). There were additional major differences observed between the two immunoassays when amino acid substitutions were made specifically in the RBD of the B chain of H2 relaxin. Thus, when arginine was replaced by citrulline at B13 and/or B17, or alanine at B17, the R6 porcine relaxin RIA no longer recognized these peptides, whereas the substituted forms still showed cross-reactivity in the ELISA (see peptide numbers 3–6 in Table III). Importantly, H1 relaxin is active in the relaxin receptor binding assay (2), whereas the citrulline and alanine substituted compounds are not. Finally, when the relaxin RBD was substituted on the insulin B chain and a glycine substituted at A10 of its A chain, the resultant molecule cross-reacted strongly in the R6 porcine relaxin RIA, but not in the human ELISA (peptide number 8 in Table III). This "GRER" insulin also binds to the relaxin receptor (2).

Discussion

The present study had three main objectives: Using a homologous human relaxin ELISA, to confirm in a larger patient population our previous report (13) that relaxin concentrations were elevated in women with IDDM; to better characterize the specificities and epitope selectivity of the previously used R6 porcine relaxin RIA and the human H2 relaxin ELISA; and to seek clues as to the relevance of elevated serum relaxin concentrations in diabetic pregnancy. Although the new data confirm and extend the previous findings that serum relaxin is elevated in pregnant women with IDDM (13), they do not shed light on the possible consequences of this phenomenon on pregnancy outcome. However, the results do raise the possibility that another relaxin-like molecule may be secreted by the diabetic women, in addition to the normally secreted H2 relaxin gene product.

The relaxin concentrations obtained on serum samples from normal and type 1 diabetic pregnant women with the R6 porcine RIA in the present work are very similar to those reported in our previous study (13), and the results obtained on the normal pregnancy samples using the human ELISA are similar to those reported by Lucas *et al.* (11), who devised that assay. Serum relaxin has not previously been measured in type 1 diabetic women using the homologous human H2 ELISA.

Within each assay system, serum relaxin concentrations were significantly ($P < 0.01$) higher in the pregnant women with type 1 diabetes than in the normal pregnant controls, confirming and extending the previous observations (13). However, the mean increase in relaxin concentration in diabetic women over that of control was significantly greater (271%; $P < 0.001$) when the RIA was used than when the ELISA was used (44%; $P < 0.01$). This large difference suggests that something other than H2 relaxin (the only relaxin thus far found in the circulation during normal pregnancy) may be present in the serum of pregnant women with IDDM. That this substance cross-reacts with the rabbit anti-porcine relaxin antiserum R6 used in the RIA, but not with the goat anti-H2 relaxin antiserum used in the ELISA, suggests that it could be an abnormal relaxin, possibly that produced by the H1 gene, or even the newly discovered H3 gene that differs markedly in sequence from both H1 and H2 relaxins in all but the RBD of the B chain (3).

The present data show that anti-porcine relaxin antiserum R6 cross-reacts equally well with H1 and H2 relaxins, whereas only H2 relaxin is measured in the ELISA. (R6 also cross-reacts with the H3 gene product; R. Bathgate, personal communication.) It can be argued that the unknown substance might be a metabolite of H2 relaxin. However, antiserum R6 reacts primarily with the RBD of relaxins of all species thus far tested (16), and also requires intact di-

sulfide linkages between the A and B chains (16) and a proper three-dimensional configuration to bind the molecule effectively (2). Furthermore, a minimally metabolized H2 relaxin might be expected to retain its ability to bind to the goat anti-human H2 relaxin antiserum used in the ELISA. This question can only be resolved by isolating and sequencing the putative relaxin variant from the serum of pregnant type 1 diabetic women.

Although human pregnancy can proceed to normal-term delivery in the absence of circulating relaxin (18), there is a growing body of evidence that abnormal elevations in serum relaxin concentrations are associated with unfavorable pregnancy outcomes. Prior experiments conclusively showed excess relaxin to exert detrimental effects on pregnancy in several animal species (see Ref. 19 for review). Recent clinical studies in women whose pregnancies were induced by menotropins or clomiphene show a strong association between elevated serum relaxin and a high risk for prematurity (20) not necessarily related to multiple pregnancies (21, 22). Elevated relaxin levels may also be observed in onset of preterm ("premature") labor requiring tocolytics, or by cervical incompetence requiring placement of a cerclage (18). Most recently, Vogel *et al.* (23) reported an association between elevated serum relaxin during the 18th gestational week and preterm delivery in a group of unselected normal, healthy primiparous women. Thus, there is clearly an association between elevated circulating relaxin and prematurity, but the mechanism(s) responsible are unknown.

In view of the foregoing, the markedly elevated serum relaxin concentrations seen in some of the pregnant type 1 diabetic women could contribute to the increased risk for premature births anticipated in such subjects (see Ref. 24 for review). That we found no statistical correlation between relaxin levels and prematurity is likely due to the relatively small number of patients studied. To illustrate, there were 11 (0.7%) very early deliveries (<35 weeks) associated with significantly elevated serum relaxin among 1545 (otherwise) normal pregnancies monitored by Vogel *et al.* (23). Thus, if the rate of relaxin-associated very early prematurity in IDDM pregnancy was even twice that of normal women, the chances of observing a single case among our group of type 1 diabetic women would be less than 1 in 60.

Mean serum relaxin concentrations fell with advancing pregnancy in type 1 diabetic women, but on average remained significantly elevated above those of normal women at all stages of gestation. Although many of the individual relaxin levels observed in diabetic women were within the normal range (around 1 ng/ml), there was a spectrum of values ranging from 0.6 to 3.5 ng/ml or greater. This was similar to the distribution of birth weights, although there was no significant correlation of relaxin concentration with birth weight. Similarly, many type 1 diabetic women have infants with normal birth weight even though the group as a whole has an increased mean birth weight (24).

There appeared to be no simple explanation such as poor control or bad outcome (including malformation/perinatal death) that distinguished those type 1 diabetic women with abnormal relaxin. A lack of statistical significance was found when correlating relaxin levels with other variables. One must bear in mind that the power of the study may be a limiting factor (e.g., relaxin versus insulin dose, $r = -0.143$, $n = 164$, $P = 0.066$, power 45%; relaxin versus birth weight $r = 0.015$, $n = 81$, $P = 0.89$, power 3%). Even when a correlation reaches 1% significance, the power is only about 50%, regardless of the sample number.

The pattern of secretion of relaxin in diabetic as compared with normal pregnancy was unlike that of hCG and estradiol-17β, which were only elevated in the third trimester (14). Fetal size and growth were also only increased in type 1 diabetic women in later pregnancy. To explore a possible relationship of elevated relaxin levels to bad outcome of pregnancy, data on two pregnancies with early fetal loss, three pregnancies with late intrauterine death, and two pregnancies with fetal anomaly were arbitrarily lumped together. However, no significant relationship between serum relaxin and a generally bad outcome was detected. Clearly, greater numbers of patients in specific bad outcome groups would be needed to detect possible significant untoward effects of hyper-relaxinemia. Thus, the hypothesis (25) that relaxin is implicated in the increased risk of abnormal development of the fetus in pregnant type 1 diabetic women is neither supported nor refuted by our data. In this regard, it may be important to measure relaxin early in the first trimester when organogenesis occurs.

Lastly, the present data suggest the possible secretion of an abnormal relaxin in addition to H2 relaxin in serum of pregnant women with IDDM, and work is in progress to isolate and identify this putative variant. If indeed such an abnormal relaxin exists, its biological actions and how they might differ from those of H2 relaxin may provide new insights into the physiological significance of elevated serum relaxin levels in diabetic pregnancy.

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