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Insulin-Like Growth Factors and Insulin-Like Growth Factor Binding Proteins in Porcine Serum and Milk throughout Lactation

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ABSTRACT

IGF-I, IGF-II, and IGF binding proteins (IGFBP) were characterized in porcine serum, colostrum, and milk on d 1–28 postpartum. IGF-I and -II were measured by heterologous RIA. Serum IGFBP were characterized by Western ligand blotting and milk IGF binding activity by [^{125}I]-IGF binding assay. IGF-II accounted for 70–85% of serum IGF and rose 2-fold between d 1 and d 28. Serum IGF-I was unaffected by duration of lactation. Milk IGF-II concentrations were higher than IGF-I concentrations on d 1–7 postpartum. After d 10, milk IGF-I and IGF-II contents were not significantly different. Serum contained IGFBP with M_r of 43, 39, 34, 28, and 24 kD. Over the course of lactation, the 43- and 39-kD bands increased, the 24-kD band decreased, and the 34- and 28-kD bands were unchanged. Milk IGF binding activity increased between d 1 (28%) and d 3 (44%), then declined until d 28 (7%). Serum and milk were separated by isoelectric focusing into 20 fractions, across a gradient from pH 3 to 10, that were screened for IGFBP by Western ligand blotting. Milk contained six IGFBP of similar M_r as serum IGFBP; however, the relative amounts of the IGFBP and their apparent isoelectric points differed. In conclusion, porcine milk contains both IGF-I and -II, with IGF-II predominating. Several IGFBP with similar M_r as those found in serum are present in milk. IGF peptide concentrations were highest in prepartum secretions and colostrum, whereas IGF binding activity peaked on d 4 of lactation. (***Pediatr Res* 36: 159–168, 1994**)

Abbreviations

IGFBP, insulin-like growth factor binding protein
TBS, Tris-buffered saline

IGF-I and IGF-II have been found in the milk of all species studied (1–5). IGF seem to be intricately involved in successful lactation, as evidenced by their roles in stimulating mammary growth (6), differentiation, and galactopoiesis (7). In addition to supporting lactation, milk IGF have been postulated to exert a direct trophic effect within the gastrointestinal tract of the suckling neonate (8, 9). Very little IGF is found in the free state in physiologic fluids; rather, IGF-I and -II in serum and other extracellular fluids are associated with six genetically distinct but structurally related IGFBP (IGFBP-1 through IGFBP-6) (10). Several IGFBP are posttranslationally modified by serine phosphorylation and N- or O-linked glycosylation. Posttranslational modifications alter the net charge of the IGFBP and may also affect the affinity of the IGFBP for IGF (10). IGFBP-3, the predominant serum IGFBP, circulates as a 150-kD complex that is impermeable to vascular epithelium (11), thus increasing the half-life of circulating IGF (12). In addition, *in vitro* work has demonstrated that IGFBP modulate the interaction of IGF with its cellular receptors (13). Several IGFBP (IGFBP-3, -2, -1, and -4) have been reported in human, rat, goat, pig, and cow milk (1–5). The molecular forms of the IGFBP present in milk seem to be species specific; however, IGFBP-2 has been consistently reported in milk. Whether IGFBP in milk protect milk-borne IGF from degradation or modulate IGF action within the neonatal gastrointestinal tract is unknown.

IGF-I and IGF binding activity have been reported in porcine milk (3, 9); however, the presence of IGF-II and characterization of the specific IGFBP contained in pig milk had not been investigated. Therefore, the goal of the current study was to establish normative porcine colostrum and milk IGF-I and -II concentrations as part of our research into the role of IGF in neonatal intestinal development. In addition, determining the IGFBP profile in milk is essential to understanding what role IGFBP may play in modulating the action of IGF in milk.

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METHODS

IGF. Recombinant human IGF-I and IGF-II were generously provided by Genentech (So. San Francisco, CA) and Eli Lilly (Indianapolis, IN), respectively. 3-[^{125}I]iodotyrosyl IGF-I [thr^{59}] and 3-[^{125}I]iodotyrosyl IGF-II were purchased from Amersham, Inc. (Arlington Heights, IL).

Biologic samples. Milk and serum samples were collected between 0700 and 0900 h on d 1–4, 7, 10, 14, 18, 21, 24, and 28 of lactation from Yorkshire-Duroc crossbred sows (n = 4) housed at the Swine Research Center on the University of Illinois campus. Because parity (5) affects milk IGF concentrations, we limited our study to second-parity sows. Because of early weaning, one sow was not sampled on d 28. To collect samples, piglets were removed from the pen and sows received injections of 200 mU of oxytocin; milk was collected by manual expression within 15 to 30 min. Sows were then restrained, and 5 mL of blood were drawn from an ear vein. Serum was obtained by allowing blood to clot for approximately 30 min on ice, followed by centrifugation at 12 000 × g for 15 min. To investigate prepartum and postweaning changes in milk and serum IGF and IGFBP, prepartum mammary secretions were collected from sows (n = 4) ≤12 h before farrowing, and blood samples were collected from sows (n = 4) 6 d postweaning. Samples were stored at −70°C. Animal procedures were approved by the University of Illinois Laboratory Animal Care Committee.

Milk protein quantitation. Protein content was determined using a modified Lowry protein assay (14) with BSA (Sigma Chemical Co., St. Louis, MO) as the protein standard. Prepartum mammary secretions were diluted 1:300 and colostrum and milk samples 1:100 before assay.

Column chromatography. To dissociate the IGF from the IGFBP, serum and milk samples (500 μL) were chromatographed in 0.2 M formic acid on a 0.9 × 100-cm column containing Sephadex G-50 (fine) (Pharmacia Fine Chemicals, Piscataway, NJ) (15). Serum was applied directly to the column, and only the IGF peptide fraction (46 to 71 mL) was collected. Milk proteins were precipitated before application to the column by the addition of 500 μL of 0.2 M formic acid and centrifugation at 12 000 × g for 1 min. The entire resulting supernatant was then applied to the column. No IGF or IGF binding activity was detected in the protein pellet by RIA or [^{125}I]IGF-I charcoal binding assay, respectively. Both the milk IGFBP fraction (28–45 mL) and the milk IGF peptide fraction were collected. Eluent containing the IGFBP was collected into 50-mL tubes, adjusted to a final concentration of 200 mM Tris, 0.002% sodium azide, pH 7.4, and stored at 4°C until use in the charcoal binding assay. Fractions containing the IGF peptide were collected in 50-mL tubes containing 0.25 mL of IGF RIA buffer (0.03 M sodium phosphate, 0.25% BSA, 0.02% sodium azide, pH 7.5) and were frozen and lyophilized (Flex-Dry, FTS Systems, Stone Ridge, NY) for IGF-I and -II RIA as described below. Recovery of IGF from the column, determined by spiking several milk samples with 100 ng/mL IGF-I, was >90%.

IGF-I and -II RIA. Lyophilized IGF peptide fractions were resolubilized in RIA buffer without added BSA. Both IGF-I and -II were measured using heterologous RIA; however, acid-chromatographed pig serum and milk samples gave parallel displacement curves to the human recombinant IGF-I and -II standards (data not shown). IGF-I content was measured using [^{125}I]human IGF-I as radioligand and a polyclonal anti-somatomedin-C/IGF-I antibody generated by Drs. Underwood and Van Wyk, University of North Carolina at Chapel Hill and distributed through the National Hormone and Pituitary Program. The lowest level of detection for the assay is 25 pg/tube. The following dilution factors were used: serum and prepartum mammary secretions, 1:100; colostrum (lactation d 1–4), 1:50; and milk, 1:10. Interassay and intraassay coefficients of variation for the IGF-I assay were 7% and 4%, respectively. IGF-II content was measured by RIA using recombinant [^{125}I]IGF-II and an MAb against rat IGF-II (Mitsubishi International Corp., Philadelphia, PA). The lowest level of detection for the assay is 50 pg/tube. The following dilutions were used: serum and prepartum mammary secretions, 1:50; colostrum (lactation d 1–4), 1:20; and milk, 1:4. Interassay and intraassay coefficients of variation for the IGF-II assay were 8% and 6%, respectively.

SDS-PAGE and Western ligand blot analysis of serum IGFBP. Molecular forms of IGFBP were characterized by Western ligand blotting using the method of Hossenlopp *et al.* (16) as previously described (1, 2). Serum samples (4 μL) were mixed with Laemmli sample buffer (17) and heated at 100°C for 5 min. Samples were separated on SDS-PAGE gels consisting of 4% stacking gels and 12% separating gels. Gels were run at 70 V overnight at 4°C. Size-separated proteins were electrotransferred to nitrocellulose (0.45 μm, Micro Separations Inc., Westborough, MA) using a Labconco semidry transfer apparatus (Labconco Corp., Kansas City, MO). Nitrocellulose membranes were sequentially incubated with TBS (0.15 M sodium chloride, 0.01 M Tris-HCl) containing 3% Nonidet P-40 (Tergitol NP-40, Sigma), TBS containing 1% BSA, and TBS containing 0.1% Tween 20 at 4°C. Membranes were then incubated overnight at 4°C with 0.5 μCi of [^{125}I]IGF-I, washed with TBS, and air dried. The IGFBP were visualized by exposure to Kodak X-Omat AR film (Rochester, NY) for 7 d at −70°C. The relative intensities of the IGFBP bands on the autoradiographs were determined using the FotoAnalyst II Imager System and Collage software (Fotodyne Incorporated, New Berlin, WI).

Rotofor separation of serum and milk IGFBP. Pig serum and milk samples were separated by solution isoelectric focusing on a Rotofor apparatus (Bio-Rad, Richmond, CA). This method was used for two reasons. First, the high protein content of porcine milk resulted in poor resolution of the molecular forms of the IGFBP by West-

ern ligand blotting. Using the Rotofor apparatus, milk IGFBP focused at a higher pH than casein allowing for clearer resolution of milk IGFBP. Second, this method also allowed for determination of the approximate pI of IGFBP in porcine serum and milk. The Rotofor apparatus consists of a cylindrical focusing chamber with buffer capacity of 55 mL. The focusing chamber is separated into 20 discrete compartments by insertion of a molded polyethylene membrane core into the chamber. The entire focusing chamber is rotated at 1 rpm, which enhances sample separation. Serum samples (0.5 mL) were separated in 50 mL of buffer containing 2% glycerol, 5% Triton X-100, and 1% ampholytes (pH range of 3–10) (Bio-Rad). Defatted milk samples (1.0 mL) were separated using the same buffer with the addition of 6 M urea to enhance solubility of casein micelles by decreasing hydrophobic interactions. Focusing was carried out for approximately 4 h at 12 W constant power. Twenty fractions were collected and their pH recorded. The fractions were placed into dialysis tubing (10 000 molecular weight cutoff; Spectrapor, Spectrum Medical Industries, Los Angeles, CA) and dialyzed overnight against PBS to remove the ampholytes. The fractions were lyophilized and resolubilized in 300 μL of 50 mM Tris buffer (pH 7.4), and 30 μL were used for SDS-PAGE. One gel was stained with Coomassie blue to show the protein separation, and the second gel was Western ligand blotted as described above.

Milk IGF binding activity. Milk IGFBP activity was measured throughout lactation using a modification of the charcoal binding assay previously described (1). Duplicate samples (100 μL) of the pH-adjusted Sephadex G-50 IGFBP fractions (1:40 sample dilution) were incubated with [^{125}I]IGF-I (≈0.005 μCi) in binding buffer (0.05 M Tris-HCl, pH 7.4, 0.02% BSA) in a 1.5-mL microfuge tube. After incubation for 2 h at room temperature, 1.0 mL of activated charcoal (0.5%, in TBS + 0.02% BSA, pH 7.4) containing protamine sulfate (0.2 mg/mL) was added. Tubes were incubated for 30 min at 4°C, then centrifuged at 12 000 × *g* for 5 min to precipitate the unbound [^{125}I]IGF-I. The supernatants containing the bound [^{125}I]IGF-I were counted for 1 min (Cobra Auto-Gamma Counter, Packard Instruments, Downers Grove, IL). The percent bound was calculated by dividing cpm recovered in the supernatants minus the nonspecific binding (7%) by the total counts added (approximately 10 000 cpm).

Statistical analysis. Statistical analyses were performed using the SAS program (SAS Institute, Inc., Cary, NC). Data from the longitudinal study (d 1–28 of lactation) were compared using a repeated-measures analysis of variance and the general linear models procedure within SAS and were considered to be statistically significant at $p \leq 0.05$ by Tukey's test. The protein content, IGF-I and -II content, and IGF binding activity of prepartum secretions were compared with d-1 postpartum samples using *t* test. Serum IGF-I and -II concentrations of 6-d post-weaning sows were compared with lactating sows using *t* test. Pearson's correlations were determined between serum and milk IGF-I and -II, serum IGF and IGFBP, and milk IGF and IGF binding activity.

RESULTS

Milk protein content. Milk protein content (mean ± SD) decreased by half between d 1 and d 2 postpartum (154 ± 4 to 75 ± 16 g/L) (Table 1). Milk protein content continued to decline until d 10 of lactation, after which concentrations were maintained at approximately 40 g/L, which is within the range of porcine milk protein content previously reported (3, 9). The protein content of prepartum mammary secretions (214 ± 17 g/L) was 40% higher than that of d-1 colostrum ($p \leq 0.05$).

Milk IGF-I and -II. Milk IGF-I and -II concentrations throughout lactation are presented in Table 1. The IGF-I (18 ± 2.9 nM) and -II (39 ± 8.6 nM) contents of prepartum secretions were approximately twice those found in d-1 colostrum (9.4 ± 1.4 and 22 ± 2.4 nM, respectively). Both IGF-I and -II declined as lactation progressed, with a 4- to 5-fold drop between d 1 and d 2 postpartum ($p \leq 0.05$). IGF-II concentrations in prepartum secretions and

Table 1. *Milk protein and IGF concentrations throughout lactation**

Day	Protein (g/L)	IGF-I		IGF-II	
		μg/L	nM	μg/L	nM
Prepartum	214 ± 17†	136 ± 22.6	18 ± 2.9†	291 ± 64.5	39 ± 8.6†
1	154 ± 4.0^a	72 ± 10.7	9 ± 1.4^a	165 ± 18.0	22 ± 2.4^a
2	75 ± 16^b	31 ± 17.1	4.0 ± 2.2^b	77 ± 49.7	10 ± 4.7^b
3	73 ± 11^b	27 ± 9.3	3.6 ± 1.2^b	50 ± 14.1	6.6 ± 1.9^c
4	66 ± 9.4^c	26 ± 9.4	3.4 ± 1.2^b	38 ± 4.9	5.0 ± 0.7^{c,d}
7	55 ± 1.5^c	11 ± 1.3	1.4 ± 0.2^c	29 ± 8.1	3.8 ± 1.1^d
10	45 ± 7.1^c	14 ± 1.9	1.8 ± 0.3^c	18 ± 3.8	2.4 ± 0.5^d
14	39 ± 7.2^c	12 ± 2.3	1.6 ± 0.3^c	16 ± 3.6	2.1 ± 0.5^d
18	40 ± 10^c	12 ± 2.0	1.6 ± 0.3^c	16 ± 1.0	2.1 ± 0.1^d
21	42 ± 10^c	10 ± 1.3	1.3 ± 0.2^c	15 ± 1.4	2.1 ± 0.2^d
24	41 ± 6.1^c	11 ± 2.7	1.4 ± 0.4^c	14 ± 4.3	1.8 ± 0.6^d
28	40 ± 5.3^c	10 ± 1.9	1.3 ± 0.2^c	11 ± 1.3	1.5 ± 0.2^d

* Mean ± SD. Prepartum, $n = 4$; d 1–24, $n = 4$; d 28, $n = 3$. Different letter superscripts indicate differences at $p < 0.05$.

† Significantly greater than d-1 postpartum samples, $p < 0.001$.

in colostrum through d 7 postpartum were approximately 2-fold higher than IGF-I concentrations. After d 10 postpartum, IGF-I and -II concentrations were similar, averaging between 1 and 3 nM (10–20 μg/L). Serum IGF-II concentrations were inversely correlated with milk IGF-II ($r = -0.46$, $p = 0.002$), whereas no relationship was observed between serum and milk IGF-I ($r = 0.02$, $p = 0.91$).

Serum IGF-I and -II. Serum IGF-I and -II concentrations throughout lactation are contained in Table 2. IGF-II is the predominant serum IGF in the sow, accounting for 70–85% of the total serum IGF. Serum IGF-II concentrations rose progressively over the course of lactation from 31 ± 7.8 nM (231 ± 58 μg/L) on d 1 to 68 ± 15 nM (511 ± 110 μg/L) by d 28 postpartum. IGF-II concentrations were significantly elevated over d-1 concentrations by d 10 postpartum. Serum IGF-I concentrations ranged between 7.0 and 18 nM (50 and 140 μg/L) and were not affected by the stage of lactation. Serum IGF-I and -II concentrations of sows 6 d postweaning (nonpregnant, nonlactating sows) were significantly higher than those on d 28 postpartum ($p > 0.05$).

Western ligand blot of serum IGFBP throughout lactation. Autoradiographs of serum IGFBP profiles for two sows throughout lactation are shown in Figure 1. Sow serum contained IGFBP with apparent M_r of 43, 39, 34, 28, and 24 kD. The 43-, 39-, 34-, and 28-kD bands have been immunologically identified (18, 19). The 43- and 39-kD bands are glycosylated variants of porcine IGFBP-3 (18); the 34-kD band is IGFBP-2, a nonglycosylated IGFBP (18, 19); and the 28-kD band is IGFBP-1 (19). The 24-kD band has not been immunologically identified in porcine serum but may be IGFBP-4, on the basis of its molecular weight, which is similar to that of rat and human IGFBP-4 (10). IGFBP-4 can also be found as a 28-kD glycosylated protein (20), potentially contributing to the 28-kD band in porcine serum.

Densitometric analysis of the relative intensities of the serum IGFBP throughout lactation for all four sows is shown in Table 3. To correct for variation between sows, the intensity of each of the IGFBP bands was normalized by the d-1 postpartum sample for each sow. The intensities of the 43- and 39-kD bands tended to increase during

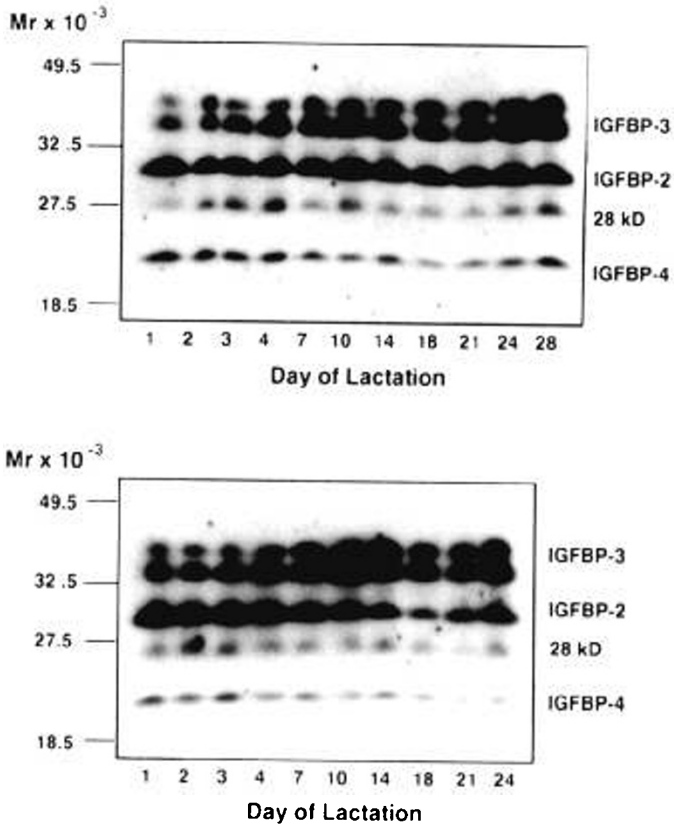

Figure 1. Representative serum IGFBP profiles throughout lactation. Autoradiographs of Western ligand blots of serum IGFBP profile of two sows throughout lactation. Serum samples (4 μL) were separated by SDS-PAGE, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 7 d. Bands with apparent M_r of 43, 39 34, 28, and 24 kD were visualized. The intensity of the 43- and 39-kD bands tended to increase during lactation, whereas the intensity of the 34- and 24-kD bands declined. No significant trend was observed in the expression of the 28-kD IGFBP.

Table 2. *Serum IGF concentrations throughout lactation**

Day	IGF-I		IGF-II	
	μg/L	nM	μg/L	nM
1	57 ± 20	7.4 ± 2.7^{a}	231 ± 58	31.0 ± 7.8^{a}
2	72 ± 25	9.4 ± 3.3^{ab}	282 ± 84	37.9 ± 11.2^{a}
3	87 ± 35	11.4 ± 4.6^{b}	305 ± 85	40.8 ± 11.5^{a}
4	136 ± 37	17.8 ± 4.9^{c}	312 ± 120	41.7 ± 7.1^{a}
7	60 ± 16	7.8 ± 2.0^{a}	286 ± 63	38.2 ± 8.7^{a}
10	60 ± 14	7.8 ± 1.9^{a}	387 ± 103	51.8 ± 13.8^{b}
14	107 ± 44	14.0 ± 5.7^{d}	407 ± 106	54.5 ± 14.1^{b}
18	50 ± 12	6.5 ± 1.6^{a}	415 ± 81	55.5 ± 10.8^{b}
21	51 ± 6.8	6.6 ± 0.9^{a}	462 ± 122	61.9 ± 16.3^{bc}
24	57 ± 9.1	7.4 ± 1.2^{ab}	492 ± 17	65.8 ± 2.2^{c}
28	53 ± 3.6	7.0 ± 0.5^{ab}	511 ± 110	68.4 ± 14.7^{c}
NPNL	72 ± 11	9.4 ± 1.4^{b}	546 ± 57	73 ± 7.6^{c}

* Mean ± SD. d 1–24, $n = 4$; d 28, $n = 3$. Different letter superscripts indicate differences at $p \leq 0.05$. NPNL, nonpregnant, nonlactating sows, 6 d postweaning ($n = 4$).

Table 3. *Densitometric analysis of serum IGFBP throughout lactation**

Day	IGFBP from Western ligand blot			
	43 and 39 kD†	34 kD	28 kD	24 kD
1	100‡^{a}	100^{a}	100^{a}	100^{a}
2	144 ± 41^{ab}	80 ± 33^{ab}	165 ± 95^{a}	109 ± 77^{a}
3	155 ± 56^{ab}	77 ± 41^{ab}	148 ± 89^{a}	80 ± 18^{a}
4	176 ± 69^{b}	71 ± 35^{ab}	151 ± 125^{a}	56 ± 12^{b}
7	182 ± 85^{b}	78 ± 43^{ab}	113 ± 44^{a}	58 ± 22^{b}
10	166 ± 98^{ab}	67 ± 43^{b}	147 ± 77^{a}	48 ± 10^{b}
14	211 ± 93^{b}	77 ± 40^{ab}	98 ± 61^{a}	41 ± 35^{b}
18	174 ± 148^{ab}	73 ± 22^{ab}	88 ± 26^{a}	21 ± 10^{b}
21	195 ± 87^{b}	78 ± 27^{ab}	79 ± 13^{a}	19 ± 17^{b}
24	209 ± 103^{b}	90 ± 49^{ab}	127 ± 67^{a}	24 ± 24^{b}
28	261 ± 127b^{b}	73 ± 43^{ab}	173 ± 140^{a}	71 ± 24^{ab}

* Mean ± SD. d 1–24, $n = 4$; d 28, $n = 3$. Different letter superscripts indicate differences at $p \leq 0.05$.

† The intensities of the 43- and 39-kD bands were summed.

‡ Data for each sow were normalized by the intensity of the IGFBP band on d 1 of lactation.

lactation and were significantly greater than d-1 postpartum intensities on d 4, 7, 14, and 21–28 postpartum. The intensity of the 34-kD band averaged 70% of the concentrations observed on d 1 postpartum but was only significantly lower on d 10 of lactation. The amount of the 28-kD band in serum varied during lactation and was not significantly different from d 1 at any other time during lactation. The intensity of the 24-kD band showed a marked decline as lactation progressed and was significantly lower than d-1 values after d 3 postpartum ($p \leq 0.05$).

Rotofor separation and Western ligand blotting of serum and milk IGFBP. Rotofor separations of d-4 postpartum porcine serum and milk samples are shown in Figures 2 and 3, respectively. Each figure shows duplicate gels: one was stained with Coomassie blue (*panel A*) and one was ligand blotted with [^{125}I]IGF (*panel B*). In serum, the 43- and 39-kD IGFBP focused between pH 5.3 and 6.5, whereas the lower molecular mass IGFBP (34, 28, and 24 kD) focused above pH 6.5 (Fig. 2*B*). The 43- and 39-kD bands (IGFBP-3) focused in the same fractions as serum albumin (Fig. 2*A*). IGFBP-2 (34 kD) and IGFBP-1 (28 kD) focused primarily in fractions between pH 6.5 and 7.5, whereas the 24-kD band was fairly evenly distributed in fractions between pH 6.5 and 9.6. Rotofor separations of serum from d 14 and 24 postpartum showed similar IGFBP abundance and separation profiles (data not shown).

Five IGFBP with M_r similar to those in serum were observed in porcine milk (d 4 postpartum) (Fig. 3*B*). However, the relative amounts of the IGFBP bands in serum and milk were not identical. Relative to serum IGFBP, the 24-kD band was less prevalent in milk than in serum, whereas the 28-kD band was present in greater amounts in milk than in serum. Several differences were noted in the Rotofor separations of porcine serum and milk IGFBP. In general, milk IGFBP focused more closely together (pH 6.6 to 7.8) than serum IGFBP (pH 5.3 to 9.6). In particular, the 43- and 39-kD bands in milk focused at more neutral pH than the serum IGFBP of similar M_r. In addition, the 28-kD band was apparent in neutral to basic fractions (pH 7.2–7.8) and in acidic fractions (pH 4.7–5.5), cofocusing with casein (Fig. 3*A*). IGFBP were barely detectable on Rotofor separations of milk samples from d 14 and 24 postpartum (data not shown).

Milk IGF binding activity throughout lactation. The results of the charcoal binding assay are shown in Figure 4. Total [^{125}I]IGF binding capacity was measured using IGFBP that had been stripped of endogenous IGF by Sephadex G-50 formic acid column chromatography. The assay was performed using 100 μL of samples of the IGFBP fraction (diluted 1:40); therefore, the total binding activity was measured in the equivalent of 2.5 μL of the original milk sample. The assay was checked for linearity using the milk sample that showed the highest total binding activity (55% specific binding). The assay was linear ($r = 0.98$) between 10- and 100-μL sample size, representing 19 to 55% specific binding of the [^{125}I]IGF-I. IGF binding activity (mean ± SD) decreased in porcine milk during lactation from 28 ± 8% on d 1 to 7 ± 1.2% on d 28 postpartum. However, unlike IGF-I and -II, which were highest in prepartum secretions and colostrum, [^{125}I]IGF binding activity rose for the first 2 d postpartum, peaking on d 3 and 4 postpartum (44 ± 9.9%) before declining. Prepartum secretions, which contained nearly twice as much IGF-I and -II as the d-1 colostrum, bound only 17 ± 4.5% of the [^{125}I]IGF-I. Milk IGF binding activity was only weakly correlated with milk IGF-I ($r = 0.30$, $p = 0.05$) and was not significantly correlated with milk IGF-II concentration ($r = 0.26$, $p = 0.09$).

DISCUSSION

IGF undoubtedly contribute to the growth-promoting activity of milk (3, 8, 21) and thus may play a role in the marked intestinal growth observed in suckling neonatal animals (22). IGF-I has been previously reported in human milk (1, 23, 24) and the milk of rats (2), pigs (3), goats (4), and cows (5). In addition, IGF-II has been measured in human (1), rat (2), ovine (25), and bovine (26) milk. This study is the first to report IGF-II concentrations in porcine milk.

IGF-II was found to be the predominant IGF in porcine serum, prepartum mammary secretions, colostrum, and milk through d 7 postpartum. Concentrations of IGF-II in porcine milk through d 7 postpartum were approximately 2-fold higher than IGF-I. On d 10 and throughout the rest of lactation, both IGF-I and -II concentrations were significantly lower than colostral concentrations and were of approximately equal concentration (10–30 μg/L or 1–3 nM). IGF-I concentrations of 30 to 140 μg/L (4 to 17.8 nM) in porcine prepartum mammary secretions and colostrum were higher than those reported in human (7–27 μg/L) (1, 23, 24) or rat colostrum (25–30 μg/L) (2), but were below concentrations in bovine colostrum (100–600 μg/L) (5). Our IGF-I concentrations were also somewhat lower than those reported for porcine colostrum by Simmen *et al.* (3, 9). They reported colostral IGF-I concentrations in the range of 67–357 μg/L (3) and 584–1271 μg/L (9). Variation between the studies may be a reflection of breed differences or the fact that their samples were collected within 12 h postpartum, whereas our first samples may have been obtained >12 h postpartum. IGF-I and -II concentrations in porcine milk after d 7 postpartum were between 10 and 30 μg/L, which is comparable to concentrations reported for other species (1–5, 9, 23–25).

IGF in milk may arise from *de novo* mammary synthesis or by transfer from the maternal circulation. IGF-I (27, 28) and IGF-II (28) mRNA have been reported in porcine mammary tissue; however, the expression is low, suggesting that maternal serum is the likely source of milk IGF. The presence of both type I and type II IGF

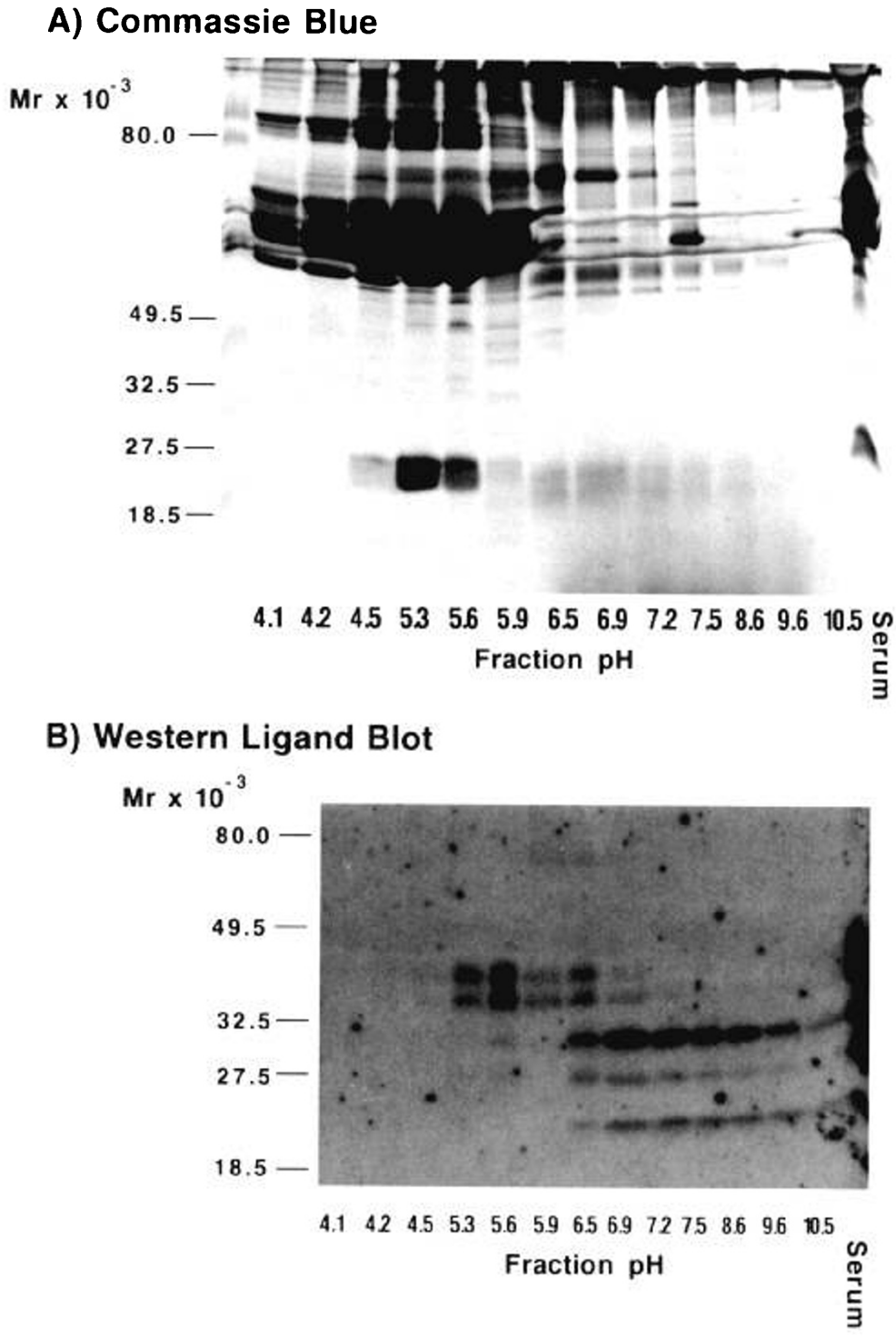

Figure 2. Rotofor separation of porcine serum from d 4 postpartum. A serum sample (500 μL) was separated by isoelectric focusing on a Rotofor apparatus into 20 fractions over a pH gradient of 3 to 10. Fractions 8–20 were separated on duplicate SDS-PAGE gels. One gel was stained with Coomassie blue to show the protein separation (*A*). The other gel was transferred to nitrocellulose, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 10 d (*B*). Bands with apparent M_r of 43, 39 34, 28, and 24 kD were visualized.

receptors (28) in porcine mammary tissue could allow for receptor-mediated translocation from the maternal circulation into milk.

Recent studies by Prosser *et al.* (25, 29) have provided direct evidence that serum is the primary source of milk IGF. Close-arterial infusions of [^{125}I]IGF-I (29) or [^{125}I]IGF-II (25) into one mammary gland of lactating goats demonstrated that both peptides are transported from the maternal circulation into milk. However, it seems that either the method of transport or the saturability of the pathway differs for the two peptides. The addition of unlabeled IGF-I to the infusate reduced the specific activity of [^{125}I]IGF-I in milk, suggesting that the IGF-I transport mechanism is competitive and saturable

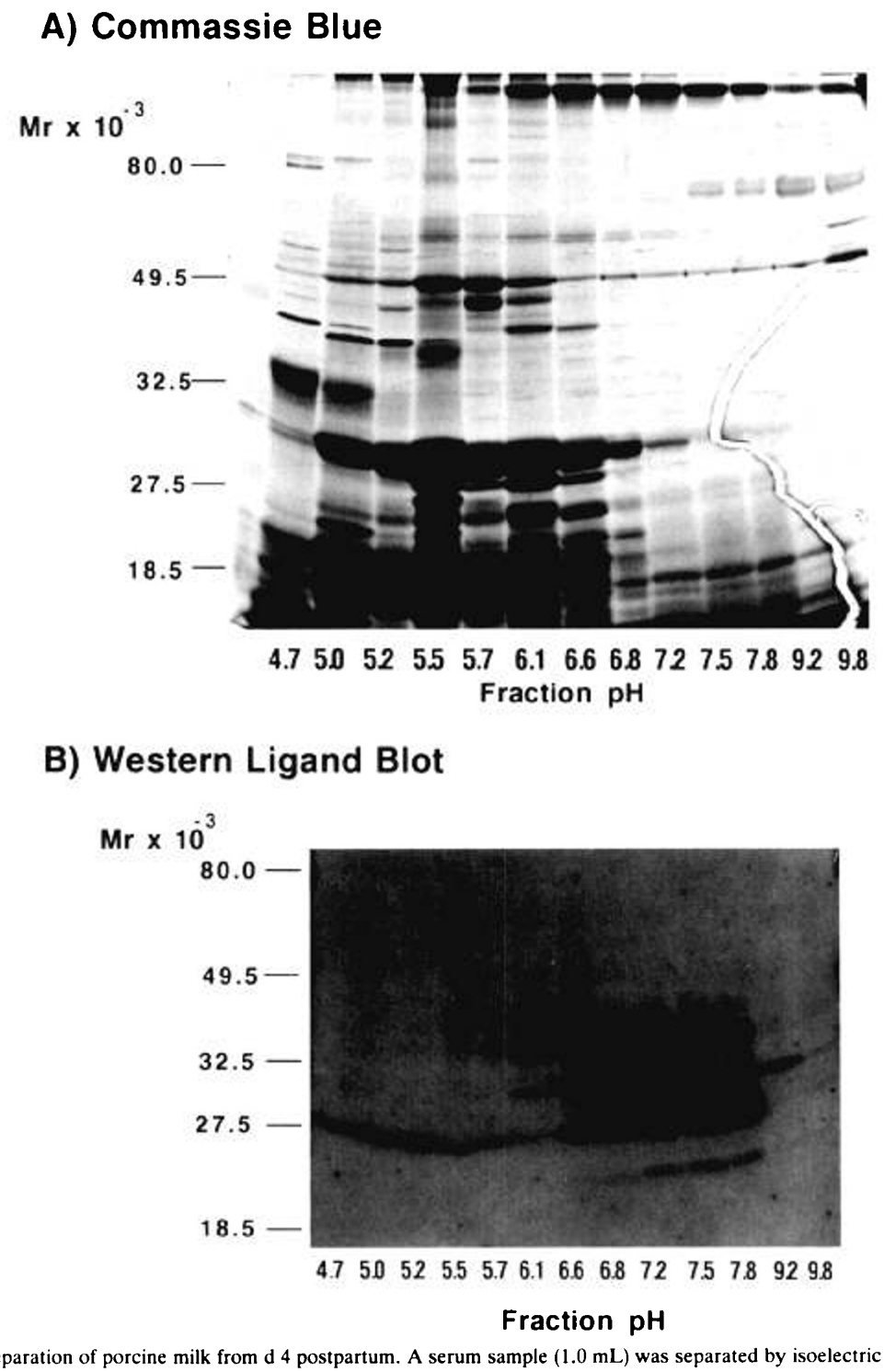

Figure 3. Rotofor separation of porcine milk from d 4 postpartum. A serum sample (1.0 mL) was separated by isoelectric focusing on a Rotofor apparatus into 20 fractions over a pH gradient of 3 to 10. Fractions 8–20 were separated on duplicate SDS-PAGE gels. One gel was stained with Coomassie blue to show the protein separation (*A*). The other gel was transferred to nitrocellulose, Western ligand blotted using [^{125}I]IGF-I, and exposed to film for 10 d (*B*). Bands with apparent M_r of 43, 39, 34, 28, and 24 kD were visualized.

(29). In contrast, the presence of unlabeled IGF-II in the infusate did not lower the specific activity of [^{125}I]IGF-II in milk (25). They concluded that IGF-I is transported into milk via a transcellular rather than paracellular route, whereas IGF-II is transported nonspecifically, potentially within pinocytotic vesicles. The inverse relationship between pig serum and milk IGF-II concentrations ($r = -0.46, p = 0.002$) supports the hypothesis that milk IGF-II is derived from the maternal circulation. In early lactation, when milk IGF-II content is at its highest, serum IGF-II concentrations are depressed. As milk IGF-II concentrations decline through lactation, a concomitant rise in circulating IGF-II concentrations is observed. The additional increase in both serum IGF-I and -II concentrations postweaning also supports this hypothesis (Table 2).

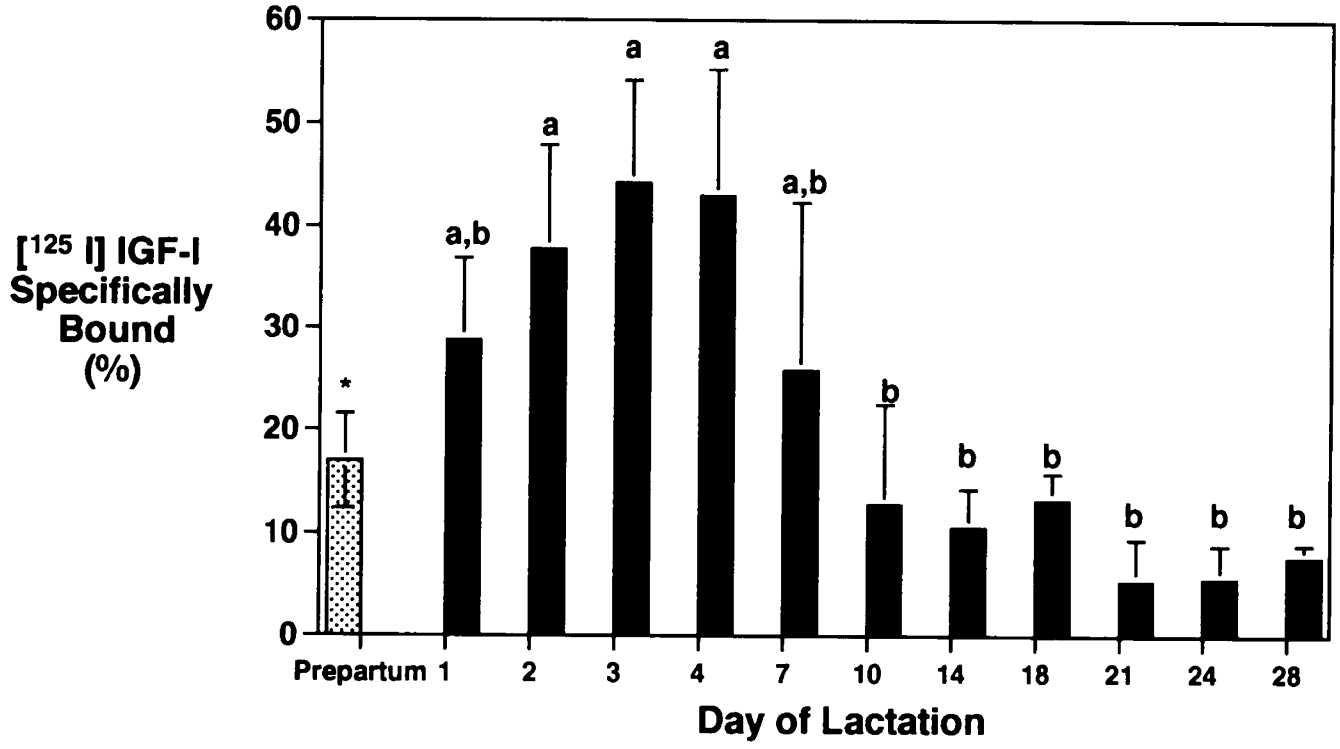

Figure 4. Pig milk IGFBP binding activity throughout lactation. Milk IGFBP activity (mean ± SD) of prepartum mammary secretions and sow colostrum and milk from d 1 to 28 of lactation was assessed by the charcoal method. Milk [^{125}I]IGF-I binding activity rose until d 4 postpartum, then declined through d 28 of lactation.

Four of the six known IGFBP (IGFBP-1 through -4) have been reported in milk. Initial studies characterized milk IGF binding activity as high molecular mass (150 kD) or low molecular mass (40 kD) IGFBP fractions by incubating milk with [^{125}I]IGF and separating the labeled peaks by gel filtration column chromatography. The 150-kD peak is the circulating molecular mass of serum IGFBP-3 and is composed of a ternary complex of IGFBP-3, IGF peptide, and an 85-kD protein known as the the acid labile subunit (11). Using the gel filtration method, the 150-kD peak (IGFBP-3) and the 40-kD binding peak were demonstrated in human (23, 24), rat (30), and porcine (3) milk. Recent studies using Western ligand blotting have characterized which IGFBP contribute to the 40-kD peak in milk. The low molecular mass IGFBP in milk vary by species but include IGFBP-2 (1, 2, 30), IGFBP-1 (31), and IGFBP-4 (2, 30).

In this study, the porcine milk IGFBP were characterized by isoelectric focusing and Western ligand blotting. Five IGFBP bands with apparent M_r of 43, 39, 34, 28, and 24 kD were detected in porcine milk. Relative to the abundance of the other IGFBP, the 24-kD IGFBP in milk was less abundant than in serum, which is similar to what we have observed in the rat (2). The IGFBP in porcine milk focused primarily at neutral to slightly basic pH with the exception of the 28-kD band, which was also present in acidic fractions (pH 4.7–5.5). If the 28-kD band is IGFBP-1, the pH change may be caused by posttranslational modifications, inasmuch as IGFBP-1 has been shown to be phosphorylated in porcine serum (32). However, the 28-kD band in milk may not be IGFBP-1, because it was not recognized by an antibody to IGFBP-1 (R. H. McCusker, personal communication). Additional purification and characterization of the IGFBP from each fraction will determine whether posttranslational modifications are responsible for the apparent pI differences between milk and serum IGFBP or whether the bands represent different IGFBP.

Longitudinal changes in milk total IGF binding activity were measured by charcoal binding assay. Interestingly, although IGF peptide concentrations were highest in prepartum secretions through d 2 of lactation, peak milk IGF binding activity was not observed until d 4 postpartum. A potential explanation for the temporal differences in peak IGF peptide and IGFBP concentrations may be that milk IGF and IGFBP arise from different sources. IGF-I and -II arise primarily from the maternal circulation (25, 29) and are concentrated in the mammary gland in late gestation (5, 28). The IGF that has been sequestered before parturition is released into the milk at the onset of lactogenesis, resulting in very high milk concentrations in early milk. In contrast, IGFBP can be transported into milk from the maternal circulation or can be synthesized *de novo* within the lactating mammary gland (28). IGFBP-2 and -3 mRNA has been demonstrated in pig mammary gland (28). On d 4 postpartum, mammary IGFBP-2 mRNA concentration was 2-fold higher than at d 30 of gestation but was equal to the concentration observed on d 112 of pregnancy (28). Because of the lack of longitudinal measure-

ments of IGFBP mRNA expression in lactating pig mammary glands, we cannot determine whether our "peak" IGF binding activity correlates with peak mammary IGFBP expression.

Ingestion of colostrum by the piglet causes a marked increase in intestinal mass and functional maturation that is thought to be due in part to the presence of milk-borne growth factors (22). The presence of type I and type II IGF receptors in the intestinal epithelium of pigs (8) suggests that IGF in milk may play a role. Several recent studies have investigated the role of IGF in the neonate (33–35). When [^{125}I]IGF-I was orally administered to suckling rats, approximately 78% of the dose was retained by the animals, predominantly in the stomach and intestinal lining (33). Studies in neonatal calves have demonstrated that [^{125}I]IGF-I added to bovine colostrum was absorbed into the blood (34). In addition, consumption of a milk replacer supplemented with IGF-I resulted in lower circulating insulin and higher circulating prolactin concentrations compared with those in calves ingesting unsupplemented milk replacers (35). Taken together, these results suggest that exogenous IGF is able to at least partially survive digestion, interact with the gastrointestinal tract, be absorbed into the blood, and affect the secretion of other hormones.

In summary, both IGF-I and -II are present in porcine milk. IGF-II concentration (10–40 nM) is approximately 2-fold higher than IGF-I concentration (4–17 nM) in prepartum secretions and colostrum. In mature milk, IGF-I and -II concentrations are approximately equivalent at 1–3 nM/L. It is important to note, however, that the kD for the type I receptor in the piglet gastrointestinal tract (≈1 nM) is within the range of normal milk IGF concentrations (8). In addition, the average milk intake over lactation is 800 mL/d (36), resulting in ingestion of approximately 9 μg/d of IGF-I and 10 μg/d of IGF-II between d 7 and 28 postpartum. Our future studies will focus on whether IGF ingested at these concentrations are of physiologic importance in neonatal growth and gastrointestinal development.

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