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Purification of Monkey Prolactin from Culture Medium: Biochemical and Immunological Characterization[1] (42304)

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Abstract. Serum-free culture medium, previously incubated with dispersed monkey pituitary cells, provided a relatively uncontaminated source for extraction and purification of 15 mg of monkey prolactin. *N*-terminal group analysis of this preparation, M21GB, produced predominantly leucine. The amino acid composition closely resembles that of both human and sheep prolactin. M21GB monkey prolactin migrates in 10% polyacrylamide with sodium dodecyl sulfate (SDS) as a single band with a molecular weight of about 23,000. Multiple bands typical of prolactins are seen with nondenaturing polyacrylamide disc electrophoresis. M21GB contains less than 1% growth hormone, incorporates radioactive iodine with a specific activity of 15 μCi/μg and specifically binds to the anti-human prolactin serum-3 provided by the National Hormone and Pituitary Program (B spec/total = 23%). M21GB does not compete in a linear fashion with iodinated human prolactin-I6 for the human prolactin antiserum, but M21GB does compete in a linear fashion with iodinated M21GB with the same antiserum. Monkey serum, pituitary homogenate, and culture medium containing unknown levels of monkey prolactin are not parallel with NIAMDD-HPrl-RP1 in the human prolactin assay, but are parallel when M21GB is used as the reference preparation and for iodination. Finally, antisera to M21GB were generated in rabbits which are specific for monkey and human prolactin and which can be used for radioimmunoassay or immunocytochemistry. In summary, serum-free medium from primary cultures of dispersed monkey pituitaries provided a quantitity of monkey prolactin which promoted biochemical analysis and production of a specific antiserum. This culture system may be a unique and ongoing source for extraction of significant quantities of monkey prolactin suitable for investigative use. © 1986 Society for Experimental Biology and Medicine.

Important differences are apparent between humans and rodents in the regulation of prolactin secretion, and nonhuman primates provide an important alternative model for research in this area. However, a reliable homologous radioimmunoassay for monkey prolactin has not been developed for widespread use, in part because of the unavailability of purified monkey prolactin. The early homologous human prolactin assay provided by the National Hormone and Pituitary Program consisted of HPrl-VLS No. 1 and anti-human prolactin serum SLV No. 1 and was validated for measurement of monkey prolactin in serum samples (1). Although the reagent preparations have changed, the human prolactin radioimmunoassay (RIA) is still widely used as it is the only means for studying prolactin regulation in monkeys. Unfortunately, there is a significant amount of nonparallelism between monkey samples and the current human prolactin standard preparation, NIH-Hprl-RP1 (AFP 2312C). In addition, samples containing high concentrations of monkey prolactin, such as medium from cultures of dispersed monkey pituitaries, will not completely displace iodinated human prolactin-I6 (AFP 2284C) from the anti-human prolactin serum-3 (AFP C11580) which is provided by NIH. The accumulation in this laboratory of quantities of spent culture medium containing large amounts of monkey prolactin afforded an opportunity to purify this hormone for use in biochemical and physiological research.

Materials and Methods. In experiments previously reported (2, 3), rhesus monkey (*Macaca mulatta*) pituitary glands were enzymatically dispersed and cultured in serum-

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free medium and on extracellular matrix for periods up to 1 month. In most experiments the serum-free medium (Ham's F-12 and Dulbecco's modified Eagle's, 1:1) contained insulin, transferrin, selenium, and cadmium plus or minus estradiol-17β. In other experiments thyroxine, parathyroid hormone, fibroblast growth factor, ethanolamine, and a mixture of lipids were also present. After determination of the prolactin concentration with the human prolactin assay provided by NIADDK, the medium was stored at −20°C for up to 3 years. Upon thawing, a pool of approximately 1.4 liters of medium was generated and employed for the purification of prolactin. An aliquot of this pool was saved for reference purposes.

Purification of prolactin was effected by precipitation with metaphosphoric acid (HPO_3) and gel filtration. The pool of culture medium (1340 ml) was adjusted to pH 4.0 by the addition of freshly prepared 0.5 *M* HPO_3 and allowed to stand at 4°C for 2 days. The resulting precipitate (M21P) was collected by centrifugation, dissolved in 6 ml of 0.2 *M* NH_4HCO_3, and applied to a 2.5 × 90-cm column of Sephadex G100 in 0.05 *M* NH_4HCO_3. A 3-ml aliquot of the supernatant (M21S) was lyophilized and set aside. The elution pattern shown in Fig. 1 revealed a large protein peak emerging from the column followed by two smaller peaks. Tubes comprising these peaks were pooled and lyophilized as follows: M21GA, tubes 31–59; M21GB, tubes 60–80; M21GC, tubes 81–110. Subsequent analysis showed that the fraction M21GB contained the bulk of the prolactin.

Small amounts of M21GA (202 μg),

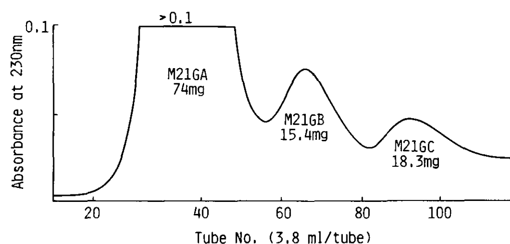

FIG. 1. Protein elution pattern from Sephadex G100 column. Proteins precipitated from cell culture medium with metaphosphoric acid were dissolved in 6 ml of 0.2 *M* NH_4HCO_3 and loaded onto a 2.5 × 90-cm column of Sephadex G100 and eluted with 0.05 *M* NH_4HCO_3.

M21GB (107 μg), and M21GC (102 μg) were dissolved in 1 ml 0.1 *M* phosphate-buffered saline (PBS, pH 7.4) for determination of prolactin content using the human prolactin RIA. The lyophilized aliquot of M21S was resolubilized in 3 ml PBS and 10 μl of M21P was diluted with 2 ml of PBS, also for prolactin assay. In addition, the growth hormone content of M21GB was determined using a human growth hormone RIA, also provided by NIADDK.

Amino acid composition was determined by the method of Spackman *et al.* (4). Samples were hydrolyzed in 5.7 *N* HCl in sealed, evacuated tubes at 105°C for 20 hr and analyzed in a Beckman Model 119 C analyzer. Cystine was determined as cysteic acid from hydrolysates of performic acid oxidized monkey prolactin. Amino terminal group analysis was performed by the dansyl technique (5, 6). Disc electrophoresis on columns of $7\frac{1}{2}$% polyacrylamide at pH 8.3 was performed by standard techniques (7).

Fraction M21GB, dissolved in PBS, was also compared to medium from a current monkey pituitary culture and to medium which had not been incubated with pituitary cells using denaturing polyacrylamide gel electrophoresis. Ten percent polyacrylamide gels containing sodium dodecyl sulfate (SDS) were prepared and the samples were mixed with loading buffer containing 0.5 *M* Tris–HCl, SDS, glycerol, 2-mercaptoethanol, and bromphenol blue prior to loading according to Dreyfuss (8). Each gel was fixed in 50% methanol/10% acetic acid, stained with Coomassie blue in methanol/acetic acid, and destained for 24 hr in 10% methanol/10% acetic acid.

Ten micrograms of fraction M21GB was dissolved in 0.5 *M* sodium phosphate buffer, pH 7.4, at a concentration of 1 μg/10 μl and mixed with 1 mCi of $K^{125}I$ (Amersham, Arlington Heights, Ill.). This was incubated with 20 μl of chloramine T (2 mg/ml) for 60–90 sec followed by addition of 60 μl of sodium metabisulfite (2 mg/ml) to terminate the reaction. PBS containing 1% gelatin (200 μl) was added before loading the mixture on a 30-cm Sephadex G100 column. The iodinated monkey prolactin was eluted with 1% gelatin-PBS. Specific binding to the anti-human prolactin serum-3 (AFP C11580, final titer 1/250,000) was tested.

Using the anti-human prolactin serum-3, comparisons were then made of the following combinations: (i) human prolactin RP-1 (AFP 2312C) and iodinated human prolactin-16 (AFP 2284C) from NIADDK; (ii) human prolactin RP-1 and iodinated M21GB; (iii) M21GB monkey prolactin for standard and iodinated human prolactin-I6; and (iv) M21GB for standard and iodinated M21GB (now referred to as the heterologous monkey prolactin assay).

Aliquots of serum from ovariectomized, lactating, and pituitary stalk-transected monkeys, as well as medium from a current monkey pituitary culture and supernatant from a homogenized monkey pituitary gland (5 ml 0.1 *M* PBS) were assayed in both the homologous human prolactin assay and with the heterologous monkey prolactin assay. Then the initial starting pool of medium and fractions M21GA and M21GC (100 μg protein/ml PBS) were assayed for prolactin content using M21GB for iodination and as the reference preparation.

M21GB was mixed with Freund's complete adjuvant and administered subcutaneously to three rabbits on four occasions. Serum samples were obtained at weekly intervals from the first innoculation and tested at multiple titers for binding capacity to iodinated M21GB. When the rabbits were killed, the binding capacity of all the samples was retested in one assay. All the bleedings from Rabbit 3 were pooled and all bleedings except the terminal sample from Rabbit 4 were pooled. Rabbit 5 had the highest titer at termination and its serum was not pooled.

Extensive characterization of the cross-reactivity with known hormones or possible contaminants of the antigen was conducted with the terminal antiserum from Rabbit 5 (anti-Mprl-5). In addition, parallelism between M21GB and numerous samples containing unknown amounts of monkey and human prolactin was determined with anti-Mprl-5. More limited examinations of the specificity of an earlier bleed from Rabbit 5, the terminal bleed from Rabbit 4, the pool of early harvests from Rabbit 4, and the pool of all harvests from Rabbit 3 were also conducted.

The preparations and concentrations used to compete with iodinated M21GB for binding to the new antisera are as follows: monkey prolactin M21GB (0.125–20 ng); National Institutes of Health (NIH) human prolactin RP-1 (0.125–20 ng); NIH rat prolactin RP-3 (1–1000 ng); NIH ovine prolactin-P-S-8 (1–1000 ng); porcine prolactin (Upjohn, 1–1000 ng); NIH human growth hormone RP-1 (1–100 ng); NIH rat growth hormone RP-1 (1–100 ng); monkey growth hormone (preparation from Dr. C. H. Li, 1–100 ng); NIH human LH-LER960 (20–10,000 ng); NIH human-FSH-HS-1 (20–10,000 ng); monkey gonadotropin preparation LER 1909-2 (20–10,000 ng); NIH human TSH-HS-4 (20–10,000 ng); NIH human chorionic gonadotropin (20–7890 ng); insulin (Sigma, 100–10,000 ng); transferrin (Sigma, 100–10,000 ng); parathyroid hormone (Peninsula, 100–10,000 ng); and fibroblast growth factor (FGF preparation from bovine brain provided by Dr. Bethea, 100–10,000 ng).

Serum samples were obtained from a cycling monkey (follicular phase), a pregnant monkey (term), a lactating monkey, a monkey with a pituitary stalk-transection, a male monkey, an ovariectomized monkey, and a pregnant human for examination of parallelism with M21GB using anti-Mprl-5. In addition, samples of culture medium from a monkey pituitary cell culture, from a monkey decidual cell culture, and from a human decidual cell culture were examined. Finally, amniotic fluid from a pregnant monkey (late gestation) and supernatant from a monkey pituitary homogenized in 5 ml PBS were also examined for parallelism.

Results. Purification of the monkey prolactin was accomplished by concentration of the proteins in the culture medium by precipitation with metaphosphoric acid followed by gel filtration. Figure 1 shows the elution pattern obtained on a column of Sephadex G100 in 0.05 *M* NH_4HCO_3. The bulk of the proteins emerge at or near the void volume of the column (M21GA, yield = 74 mg) followed by two smaller peaks: M21GB (yield = 15.4 mg) with a Ve/Vo of about 2.0, and M21GC (yield = 18.3 mg) with a Ve/Vo between 3.0–4.0. Initial RIA of the various fractions using the human prolactin assay showed a 6.6-fold concentration of prolactin in M21P and that the majority of prolactin was in fraction M21GB from the Sephadex column. Growth hormone constituted less than 1% of M21GB as deter-

mined by RIA with the NIH human growth hormone assay. Further analysis showed M21GB to contain the bulk of the prolactin with extremely minor contaminants.

Polyacrylamide disc gel electrophoresis (Fig. 2A) of the preparation shows several bands ($R_f \sim 0.6$–0.7) which is typical of many species of prolactin and growth hormone isolated from pituitary extracts (9). Further characterization was obtained by SDS-polyacrylamide gel electrophoresis. Figure 2B shows the SDS-polyacrylamide gels (denaturing conditions). Lane B contains M21GB monkey prolactin which migrated as a single band with a molecular weight of approximately 23,000. A trace of transferrin may be present. Lane C contains culture medium to which insulin, transferrin, and selenium were added, but which had not been exposed to pituitary cells. This lane does not manifest a band in the vicinity of M21GB but rather has only higher molecular weight bands. Lane D contains culture medium with the same additives, but which had been incubated with monkey pituitary cells for 4 days. This sample contains the same higher molecular weight bands seen in Lane C, and in addition, a band in the same position as M21GB is evident. Lane E contains an aliquot of monkey pituitary cells which had been in culture for 30 days and then solubilized in 20 m*M* ammonium hydroxide prior to denaturation with loading buffer. Multiple bands are evident in addition to prolactin.

TABLE I. AMINO ACID COMPOSITION OF MONKEY PROLACTIN COMPARED TO HUMAN AND SHEEP PROLACTIN

Amino acid	Monkey[a]	Human[b]	Sheep[c]
Aspartic acid	20.7	18	22
Threonine	9.7	8	9
Serine	14.4	17	15
Glutamic acid	27.6	27	22
Proline	11.4	8	11
$\frac{1}{2}$ Cystine	5.1	6	6
Glycine	9.1	7	11
Alanine	11.6	11	9
Valine	10.0	10	10
Methionine	4.7	5	7
Isoleucine	8.9	13	11
Leucine	22.7	24	23
Tyrosine	6.7	7	7
Phenylalanine	5.9	5	6
Histidine	7.9	9	8
Lysine	10.2	10	9
Arginine	10.2	12	11
Tryptophan	N.D.[d]	2	2

[a] Calculated on the basis of 197 residues per molecule.
[b] Taken from cDNA analysis, Cooke *et al.*, 1981.
[c] Protein sequence analysis, Li *et al.*, 1970.
[d] N.D., not determined.

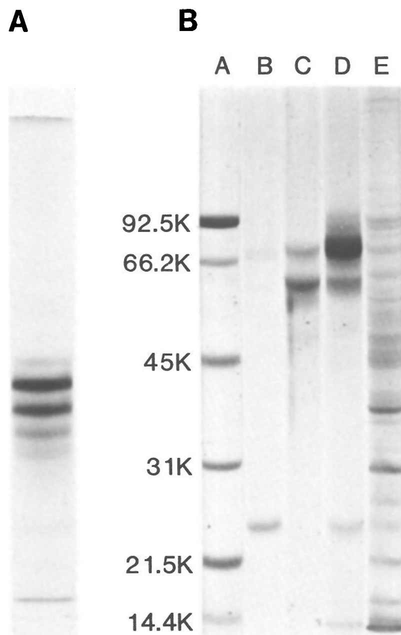

FIG. 2. (A) Polyacrylamide disc electrophoresis of fraction M21GB using $7\frac{1}{2}\%$ polyacrylamide at pH 8.3. (B) SDS–10% polyacrylamide disc electrophoresis of fraction M21GB (lane B), control culture medium with hormone additives (lane C), culture medium containing additives and incubated with monkey pituitary cells for 4 days (lane D) and solubilized monkey pituitary cells (lane E). Lane A contains molecular weight standards.

NH_2-terminal group analysis of M21GB showed leucine to be the major terminal amino acid present. Traces of phenylalanine, aspartic acid, and alanine were also detected. The amino acid composition of the monkey prolactin (M21GB) is shown in Table I and compared with the known composition of ovine and human prolactin. Monkey prolactin has a high content of aspartic acid (21 residues), glutamic acid (28 residues), and leucine (23 residues). The one-half cystine content is compatible with the presence of three disulfide bridges in the molecule. Tryptophan, which is present in other species of prolactin, was not determined (destroyed by acid hydrolysis).

Figure 3 illustrates the problems encountered when using the current homologous NIH human prolactin assay for measurement of monkey prolactin in cell culture medium. The top panel shows a typical standard curve (HPrl-RP-1) plus and minus 100 μl of culture medium and demonstrates that culture medium alone does not alter the curve. The middle and bottom panels contain standard curves versus aliquots of medium from cultures of pituitary glands from monkeys in different physiological states. In the middle panel, M46 medium (pregnant monkey) had very high levels of prolactin, and excess amounts of this medium do not completely displace the iodinated human prolactin-I6 from anti-HPrl-3, resulting in B/B_0 levels of not less than 35%. The bottom panel illustrates the results using medium of other cultures which exhibit varying degrees of nonparallelism.

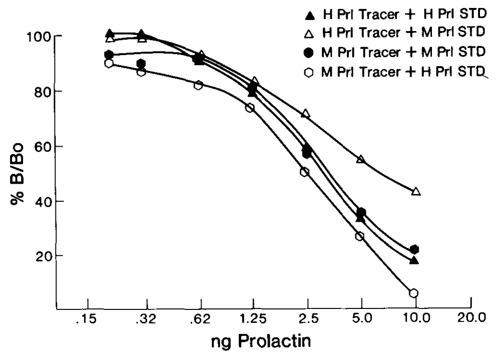

FIG. 4. Displacement of iodinated human prolactin-I6 and monkey prolactin M21GB from anti-human prolactin serum-3 by human prolactin RP-1 and monkey prolactin M21GB. The antiserum was used at a final titer of 1/250,000.

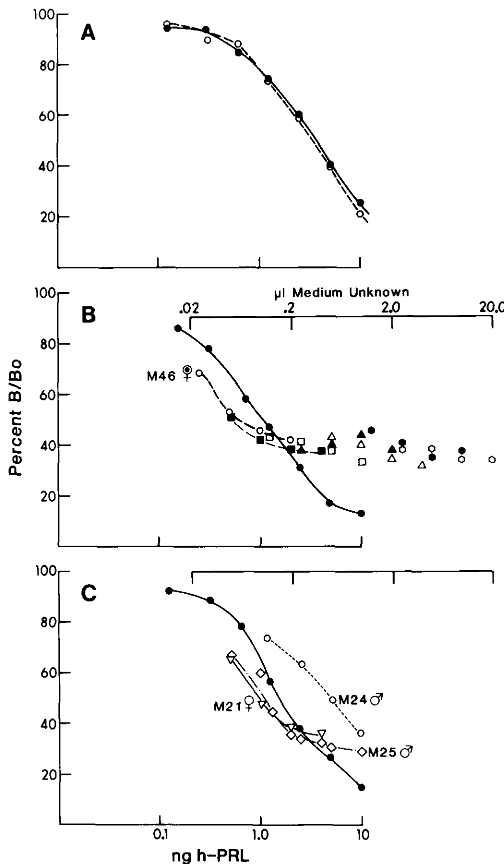

FIG. 3. Measurement of prolactin in aliquots of culture medium from various monkey pituitary cultures using the homologous human prolactin radioimmunoassay. In (A), 100 μl of culture medium (○) did not affect the standard curve (●), but in (B) and (C), culture medium incubated with monkey pituitary cells exhibited varying degrees of nonparallelism.

With the iodination conditions described, M21GB incorporated 15.5 ± 5.3% of the radioactive iodine giving a specific activity of 15 μCi/μg (n = 3). Specific binding to the anti-human prolactin serum-3 was 23 ± 2% (n = 10). Figure 4 illustrates the various competitions of human prolactin RP-1 and M21GB monkey prolactin with iodinated human prolactin I-6 and iodinated M21GB monkey prolactin. Human prolactin RP-1 displaces the iodinated human prolactin-I6 from the anti-human prolactin serum-3, as well as displacing the iodinated M21GB in a typical fashion and with similar potency. Likewise, M21GB displaces iodinated M21GB with a potency similar to that of human prolactin RP-1. However, as previously observed with culture medium, M21GB does not completely displace iodinated human prolactin I-6 from anti-HPrl-3 even at high concentrations.

Figure 5 compares the parallelism of five different samples in the homologous human prolactin assay (H-H assay) versus the assay which utilizes M21GB for iodination and as the reference preparation (M-M assay). The parallelism between the samples and the standard curve is significantly better using M21GB

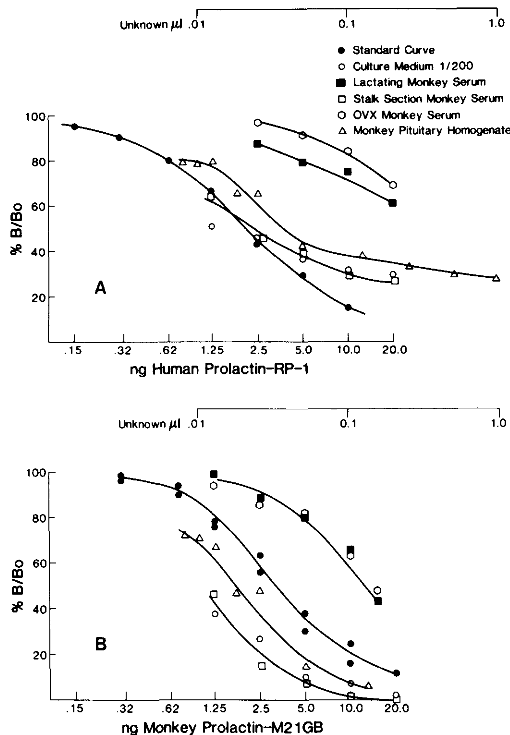

FIG. 5. Comparison of parallelism between standard preparations and samples (µl) containing unknown amounts of monkey prolactin with the human prolactin RIA (H-H assay, (A)) and with the monkey prolactin RIA (M-M assay, (B)), the latter of which utilizes M21GB for iodination and as the reference preparation.

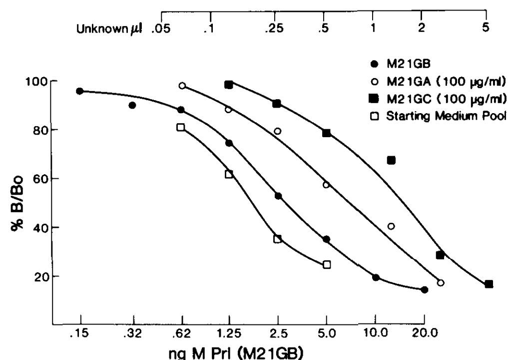

FIG. 6. Radioiodinated M21GB was displaced from the anti-human prolactin serum-3 with known quantities of M21GB versus serial dilutions of the initial starting pool of culture medium and serial dilutions of fractions M21GA and M21GC. 100 µg protein M21GA and M21GC were diluted in 1 ml PBS. The unknown scale refers to µl aliquots assayed of the culture medium and of the solubilized proteins.

as the reference preparation and iodinated tracer.

Figure 6 compares M21GB to fractions M21GA, M21GC, and to the initial starting pool with M21GB as the radioiodinated tracer. Each of these samples is parallel to M21GB. M21GA contains approximately 3.32 µg prolactin/100 µg of protein and M21GC contains approximately 2.7 µg prolactin/100 µg protein.

Figure 7 illustrates the percentage binding at a titer of 1/40,000 of each of the serum harvests from the three rabbits treated with antigen M21GB. The shaded areas illustrate which samples were pooled and/or further tested.

Figure 8 illustrates the ability of various hormone preparations to compete with iodinated M21GB for anti-monkey prolactin serum from Rabbit 5 (anti-Mprl-5). Specific binding of anti-Mprl-5 was 25.7% at a final titer of 1/200,000 using serial 24-hr incubations for first antibody, tracer, and precipitating antibody (anti-rabbit γ globulin). Monkey prolactin-M21GB and human prolactin RP-1 completely displace the M21GB tracer from anti-Mprl-5. (Although not shown here, extending these curves to 40 ng results in ~0% B/B_0).

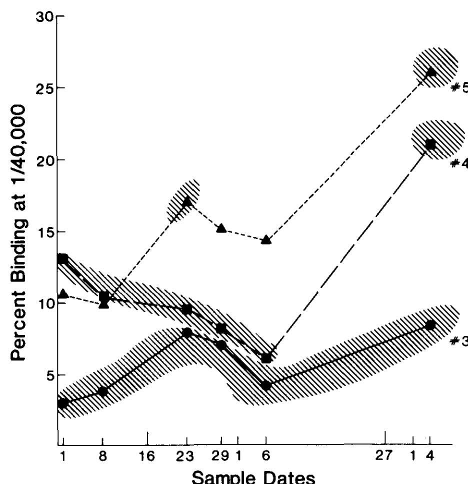

FIG. 7. Monkey prolactin antibody percentage binding of various antisera harvests, each at an initial titer of 1/40,000 (final 1/200,000), to iodinated M21GB. The shaded areas designate pools or individual samples which were tested for specificity.

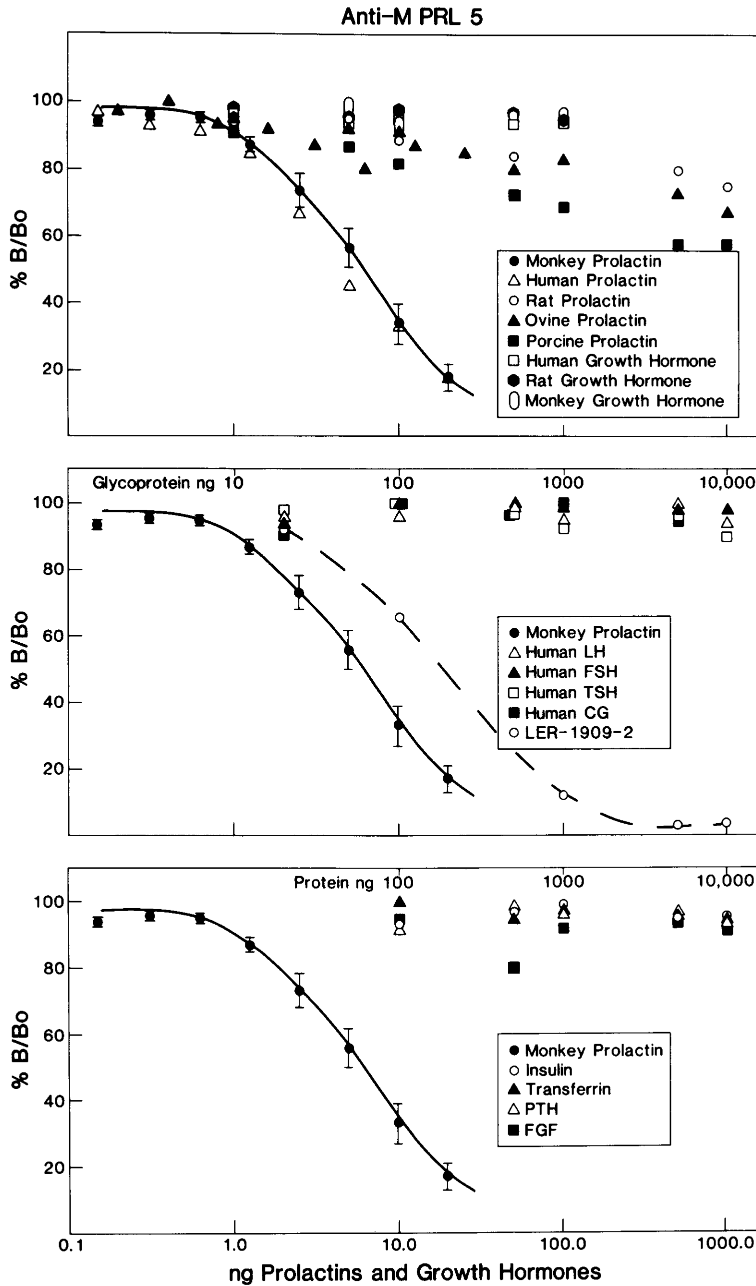

FIG. 8. Competition of various hormone preparations with iodinated M21GB for anti-monkey prolactin-5.

Porcine prolactin > ovine prolactin > rat prolactin show degrees of competition at high levels (>100 ng) suggesting that their affinity for anti-Mprl-5 is about 2–3 orders of magnitude less than monkey or human prolactin. None of the growth hormones, human glycoprotein hormones, or any of the protein additives to the initial serum-free culture medium demonstrated any cross-reaction with anti-Mprl-5. Interestingly, the monkey go-

nadotropin preparation LER 1909-2 shows excellent parallelism with M21GB suggesting that prolactin is present in this extract.

Figure 9 shows the parallelism between M21GB and various samples containing unknown levels of monkey or human prolactin using anti-Mprl-5. Both monkey and human prolactin from serum, pituitary homogenate, amniotic fluid, cultured pituitary cells, or cultured decidual cells were able to compete with iodinated M21GB for anti-Mprl-5 in a fashion parallel to M21GB standard.

Figure 10 contains the more limited tests for specificity of an earlier serum harvest from Rabbit 5 (final titer 1/200,000), and the pooled serum from Rabbit 3 (final titer 1/50,000) and Rabbit 4 (final titers 1/200,000 and 1/100,000 for pool) (see shaded areas in Fig. 7). Specific binding of about 25% was achieved at each of these titers. These results suggest that each of

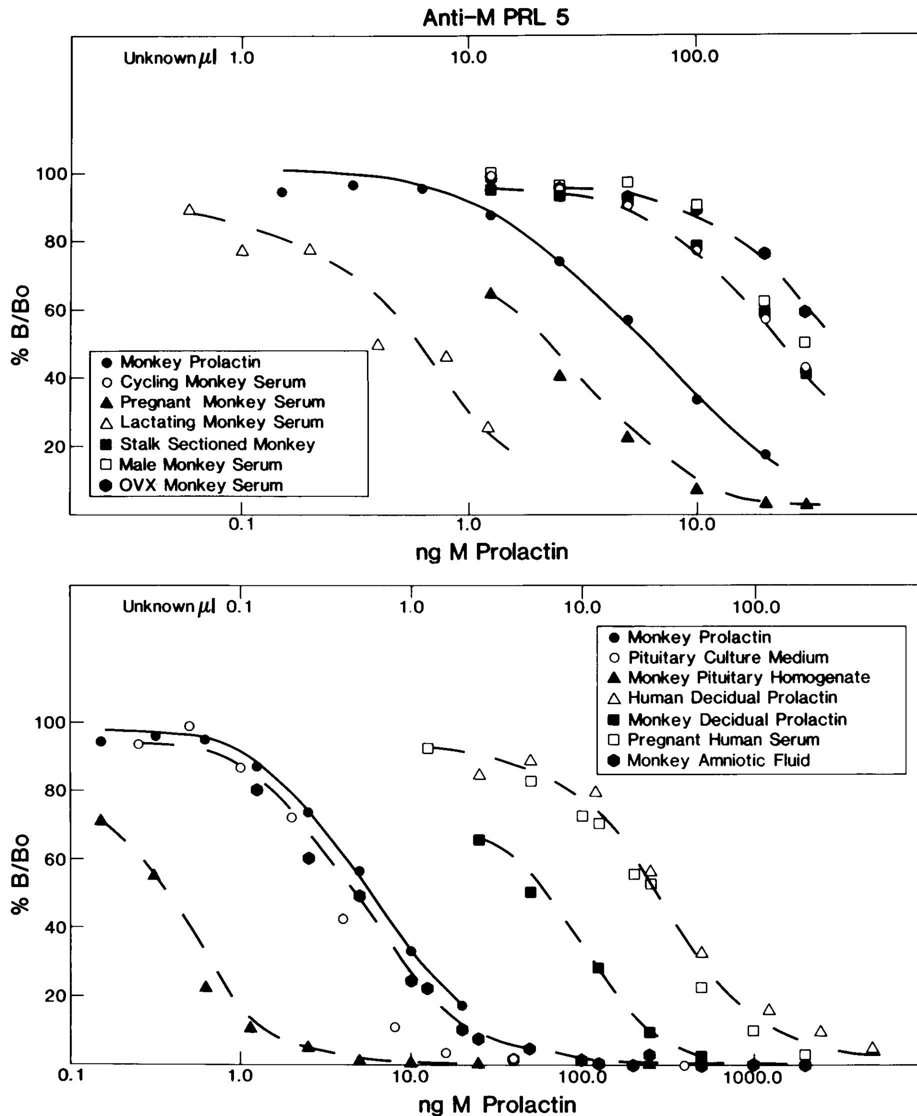

FIG. 9. Competition of samples from various sources which contain human and monkey prolactin for anti-monkey prolactin-5.

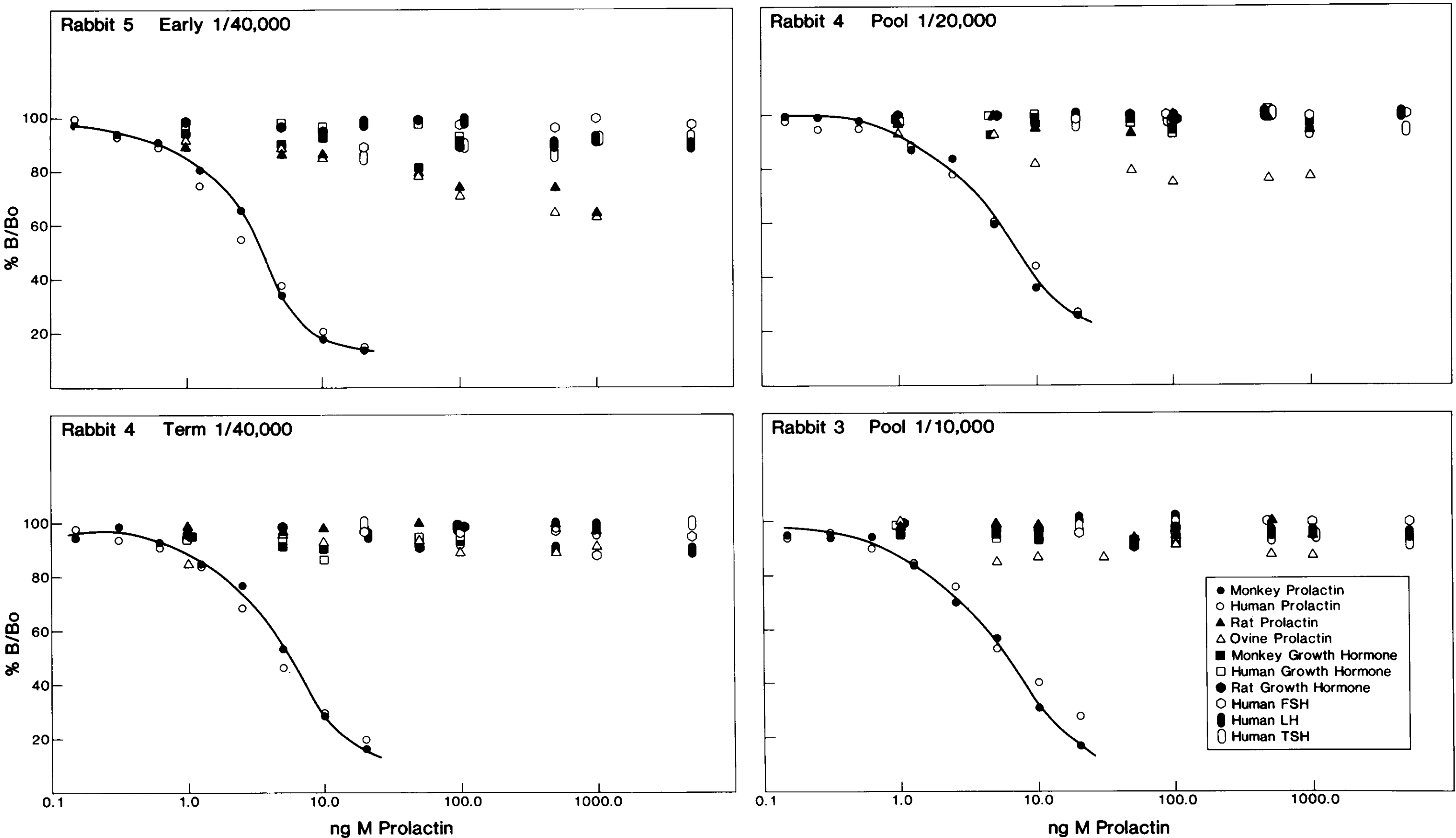

FIG. 10. Competition of various hormone preparations with iodinated M21GB for several other lower titer anti-monkey prolactin sera.

these antisera are as specific as anti-Mprl-5 and could also be used for RIA or immunocytochemistry.

Discussion. Nonhuman primates are an important model for research in reproductive endocrinology. However, measurement of the reproductive hormones in monkeys has been fraught with various problems, including the presence of a significant degree of nonparallelism when measurements of monkey prolactin are sought with the current NIH human prolactin assay.

In the course of experimentation with primary cultures of dispersed monkey pituitary cells, ~1.4 liters of serum-free culture medium was generated which contained high levels of prolactin as determined with the current NIH human prolactin radioimmunoassay. Extraction of this medium yielded ~15 mg of monkey prolactin with less than 1% growth hormone contamination and probably 0–3% of other contaminants. Two other fractions contain small amounts of prolactin and could ultimately be used for reference preparations.

The purification procedure is simple, requiring a minimum of handling, and is similar to that employed by others for the preparation of prolactin from rat and hamster pituitary culture medium (10, 11). The preparation obtained appears to be quite pure as evidenced by a single major *N*-terminal amino acid (leucine); by electrophoretic examination; and by the generation of an antiserum which does not cross-react with any of the initial additives to the serum-free medium. Further, the molecular weight estimate by SDS–gel electrophoresis (23,000) is similar to that of other mammalian prolactins. The amino acid composition is very similar to that for ovine and human prolactin with perhaps a somewhat closer resemblance to the latter with respect to several of the amino acids (glutamic, methionine, alanine) (9, 12, 13).

When the purified monkey prolactin was iodinated to a low specific activity, 23% was specifically bound by the NIH anti-human prolactin serum-3 (final titer 1/250,000). The purified monkey prolactin competes with iodinated monkey prolactin for anti-Hprl-3 in a sigmoidal fashion, but monkey prolactin does not completely displace iodinated human prolactin I-6 from the same antiserum. Thus, it is possible that this polyclonal antiserum contains species of antibodies with low affinities which were generated against an antigenic determinant in the human prolactin prepared from whole pituitaries, but this determinant is not present in monkey prolactin secreted into culture medium.

The extraction of a significant quantity of purified monkey prolactin from serum-free culture medium then allowed the production of an antibody which is specific for monkey and human prolactin. The highest titer antiserum exhibits an ED_{50} of about 4.50 ng and cross-reacts completely with human prolactin RP-1 and also with an extract of monkey pituitary, LER 1909-2. It also cross-reacts slightly with rat, ovine, and procine prolactins at high concentrations. No competition was observed with any of the growth hormones, gonadotropins, or additives originally present in the serum-free medium. The parallelism between M21GB standard and all samples of monkey serum, human serum, and culture medium containing monkey and human prolactin from pituitary and decidual cells was excellent using anti-Mprl-5. Limited tests on the other antisera of lower titer suggest they are also suitable for RIA or immunocytochemistry.

In conclusion, serum-free culture medium from primary cultures of monkey pituitary cells provided a significant quantity of highly purified monkey prolactin which allowed comparative biochemical analysis of this protein and generation of a specific antiserum. This will greatly facilitate studies of prolactin regulation in this important animal model of human reproduction. Finally, generation of prolactin-containing culture medium from primary cultures of monkey pituitary cells is an ongoing effort and may continue to be a valuable source for purification of this hormone.

The anti-serum was produced and characterized with expert guidance from Dr. William E. Ellinwood. We thank Dr. D. Chung and Dr. C. H. Li (Laboratory of Molecular Endocrinology, San Francisco, Calif.) for the amino acid and terminal group analyses, Mr. Terry Oyama and Ms. Elaine Yuzuriha for technical assistance, and Ms. Mary Anne Foley for editorial assistance.

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