PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 583–587 (1983)

Examination of Rat Placental Lactogen and Prolactin at 6-hr Intervals during Midpregnancy (41691)

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Abstract. The Nb_2 node lymphoma cell bioassay was verified in our laboratory as a sensitive, reproducible, and accurate bioassay for measurement of serum rat placental lactogen (rPL). Blood samples taken every 6 hr during pregnancy showed a significant peak of rPL secretion in the late afternoon on Day 11. The inverse relationship between rPL and nocturnal prolactin (PRL) surges is shown.

Two forms of placental lactogen (rPL) exist in the rat. Rat placental lactogen I (rPL-I) is secreted primarily on Days 8–14 and rPL-II is secreted primarily on Days 14–21 (1, 2). While various radioreceptor assays and bioassays have been used to measure lactogenic activity in the pregnant rat, only rPL-II is active in the radioimmunoassay developed by Robertson and Friesen (2). This study describes the development of the Nb_2 lymphoma cell bioassay for lactogenic hormones in our laboratory. This bioassay was first developed by Tanaka *et al.* (3). To establish a complete description of rPL secretion during midpregnancy, frequent blood samples (every 6 hr) were obtained on Days 8 through 14 of pregnancy. Serum PRL levels also were examined to investigate the hypothesis that the increasing levels of rPL cause termination of the PRL surges at midpregnancy (4).

Materials and Methods. *Animals.* Four female Holtzman rats (Holtzman Co., Madison, Wisc.) were housed with each male and kept on a 12-hr light:dark cycle, with lights on 0600–1800 hr. The morning that sperm were found in the vaginal lavage was designated Day 0. Laparotomy was performed through a midline abdominal incision under ether anesthesia on the morning of Day 7 to confirm the pregnancy. At least 8 implantation sites were required for inclusion in the study. All blood samples (0.4 ml) were taken by cardiac puncture under light ether anesthesia within 1 min of the animal leaving the cage.

Three sets of pregnant rats were used to minimize the deleterious effects of repeated cardiac punctures. The first set was bled at 0600, 1200, 1800, and 2400 hr on Day 11 only. The second set was bled at 0600 and 1800 hr on Days 8 through 14. The third set was bled at 1200 and 2400 hr on Days 8 through 14. This amount of blood removal did not affect the development of the fetuses, as evidenced by normal appearing fetuses on Day 17, when the rats were killed.

Hormone assays. The Nb_2 node lymphoma cells were obtained from Dr. Kurt Ebner, University of Kansas Medical Center, Department of Biochemistry, who originally obtained them from Dr. Noble, Dr. Beer, and Dr. Gout from the Department of Biochemistry, Faculty of Medicine at the University of British Columbia, Vancouver, British Columbia, Canada, and were maintained in tissue culture flasks in a CO_2 incubator. Fischer's medium supplemented with fetal calf serum (FCS; 10%), horse serum (HS; 10%), penicillin (50 U/ml), streptomyocin (50 U/ml), and 2-mercaptoethanol (10^{-4} M) were used to maintain the cell growth in a 5% CO_2–95% air mixture at 37°C.

One day before use in a bioassay, cell growth was slowed down by transferring the cells to the maintenance medium (described above) containing 1% FCS. On the next day, cells were washed and resuspended in a stationary medium (maintenance medium without any FCS) and 1-ml aliquots of suspended cells ($1–2 \times 10^5$ cells/ml) in the stationary medium were pipetted into 35-mm tissue culture dishes. Materials to be tested for lactogenic activity were added prior to addition of the cells. If rat serum was to be assayed for rat placental lactogen (rPL), excess antiserum to rat prolactin (50 μl) also was pipetted into each dish.

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0037-9727/83 $1.50

The antiserum initially used was NIADDK anti-rat prolactin-S-8 at a dilution of 1:1000 or a final dilution of 1:20,000. More recently NIADDK-anti-rPRL-ICF-1 was generously supplied by Dr. Parlow, and was used at a final concentration of 1:60,000. After 3 days of incubation in a CO_2 incubator at 37°C, cell numbers were determined using a Sysmex microcell counter (CC-10) obtained from American Scientific Products.

Serum samples for both assays were allowed to clot at 4°C before centrifugation and kept at −60°C until used. Dilution of peptides or serum to be assayed for growth promoting activity was done using stationary medium. Serum for rPL assay usually was diluted 1:50 and 10 and 60 μl were assayed in duplicate. Thus the maximum amount of serum usually assayed was 1.2 μl. The limit of detection of rPL in serum in this dilution was 50 ng/ml. The RIA method of Niswender *et al.* (5) was used to assay each serum sample in triplicate for PRL. The assay materials were provided by the NIADDK Hormone Distribution Program, and the reference preparation was NIADDK rat PRL-RP-1.

Statistical analysis. One-way analysis of variance was performed on serial blood samples. Duncan's multiple range *t* test was performed if the *F* statistic was significant ($P < 0.05$).

Results and Discussion. Figure 1 shows lymphoma cell number in response to increasing amounts of ovine prolactin (oPRL; NIH-S-10), rat prolactin (rPRL; NIADDK-RP-1), human growth hormone (hGH; AFP-5180A), and rat growth hormone (rGH; NIH-B-6). At doses ranging from 0.05 to 10 ng, cell response to hGH was equipotent and parallel to the response to oPRL. The rPRL preparation used was not as potent, which was expected due to its known biological activity (11 IU/mg). A purified rGH did not stimulate cell growth until 10 ng was used. Thus, rGH does not interfere in the assay since a maximum of 1.2 μl of serum is normally used. When excess antibody to rPRL was used, rPRL (10 ng) did not cause any cell growth. Only when 25 ng of rPRL was added was there any growth. This means that at serum prolactin concentrations of 8000 ng/ml or less, the amount of antiserum added to each culture dish will neutralize all the prolactin present. From these results it can be concluded that in the presence of anti-rPRL antisera, mitogenic activity is due to the presence of placental lactogen.

Figure 2 shows the results of adding increasing amounts of serum from three pregnant rats with a wide range of rPL values. Antiserum to rat prolactin was added to each well so all the growth promoting activity of

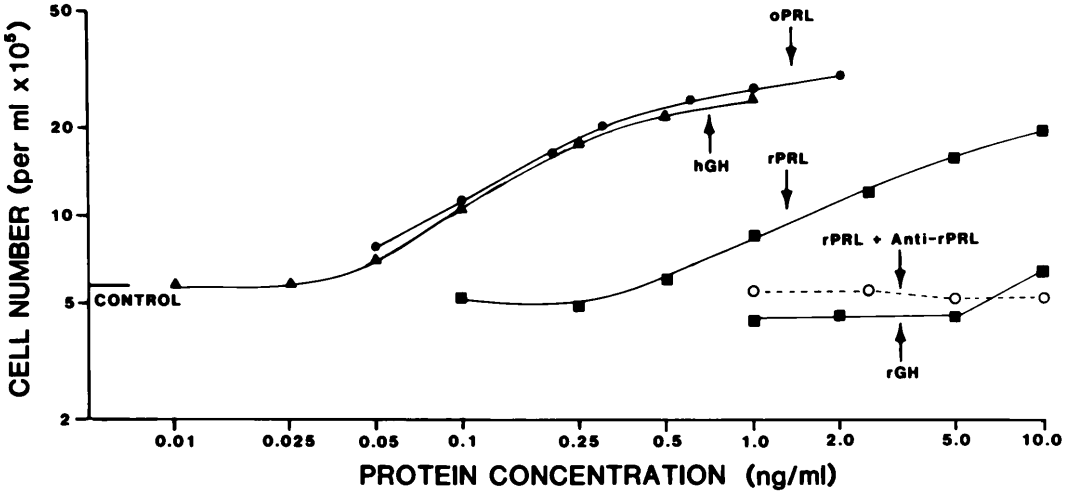

FIG. 1. Growth of Nb_2 node lymphoma cells in response to increasing doses of various prolactin and growth hormone preparations. Antiserum to rPRL was added at a final dilution of 1:60,000 where indicated. Control indicates cell number when cells in stationary medium were incubated in the absence of protein hormones. Each point is the mean of triplicate dishes incubated for 3 days.

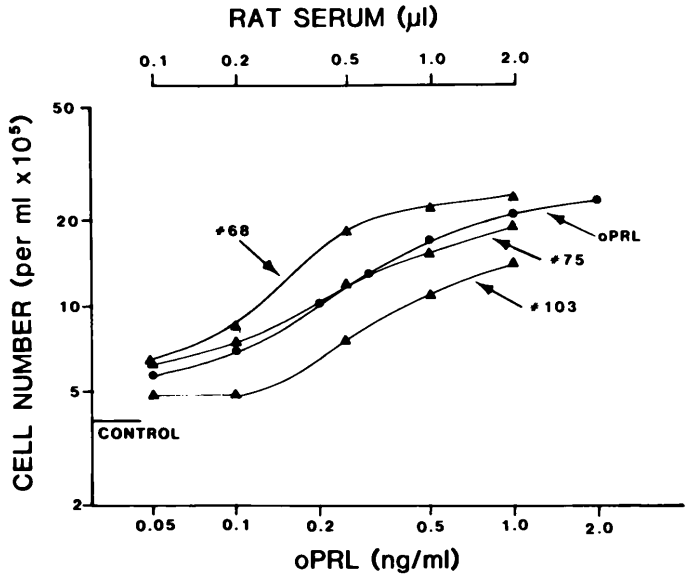

FIG. 2. Sera from three different pregnant rats or oPRL were incubated with the Nb_2 node lymphoma cells for 3 days. Antiserum to rPRL was added to each dish. Volumes of rat sera from 0.1 to 2.0 μl increased cell number parallel to that caused by increasing amounts of oPRL. Points are the mean values of triplicate dishes.

the serum was due to rPL present in the serum. All three rats showed parallel curves compared to the oPRL standard used. The coefficient of variation was 10% within and between assays for serum. Thus this bioassay for lactogenic hormones provides a means of accurately quantifying rPL levels. It does not distinguish between the two forms of placental lactogen, rPL-I and rPL-II. However, this experiment only measured levels through Day 14, at which time very little rPL-II is present (2).

Serum rPL levels (oPRL standard) for pregnant rats sampled every 6 hr during Day 11 ($N = 7$) were as follows: 3760 ± 267 ng at 0600 hr, 4290 ± 551 ng at 1200 hr, 4570 ± 734 ng at 1800 hr, and 4030 ± 389 ng at 2400 hr. No differences in mean rPL levels were found among the times of sampling on Day 11. This allowed the data from the animals bled at 0600 and 1800 hr and animals bled at 1200 and 2400 hr to be combined for statistical analysis over the days of pregnancy, as shown in Fig. 3. The first significant rise in rPL is seen at 1800 hr on Day 10. Levels continued to rise until they reach a peak at 1800 hr on Day 11, which is higher than at any other time ($P < 0.05$). Thereafter, levels declined until they reach prepeak values late on Day 14. Because rPL levels were so low on Day 14, the second form of rPL, rPL-II, which normally peaks on Day 20 (2), is not secreted in amounts large enough to interfere significantly on this day. It can be assumed therefore, that the peak seen on Day 11 is due entirely to rPL-I.

Comparison of the means for the two groups of animals bled at noon and midnight on Day 11 shows that those animals bled for the 7-day period at these hours (Fig. 3) had lower mean rPL levels than the group bled on Day 11 only. There are several possible explanations for the differences in these two groups of rats. The sampling of blood six times prior to Day 11 may have had some effect, whereas rats only bled on Day 11 had no prior exposure to ether except during laporatomy done to verify the pregnancy. Secondly, these two experiments were done several weeks apart, using two separate shipments of rats. It is interesting that rPL levels at noon on Day 10, noon and midnight on Day 11, and midnight on Day 12 are lower (not statistically) than rPL levels 6 hr before and 6 hr after these times (Fig. 3). Whether this has any biological significance or simply reflects differences due to using a separate group of rats for the noon–midnight bleedings from the 0600 to 1800-hr bleedings is not clear. In the group bled four times only

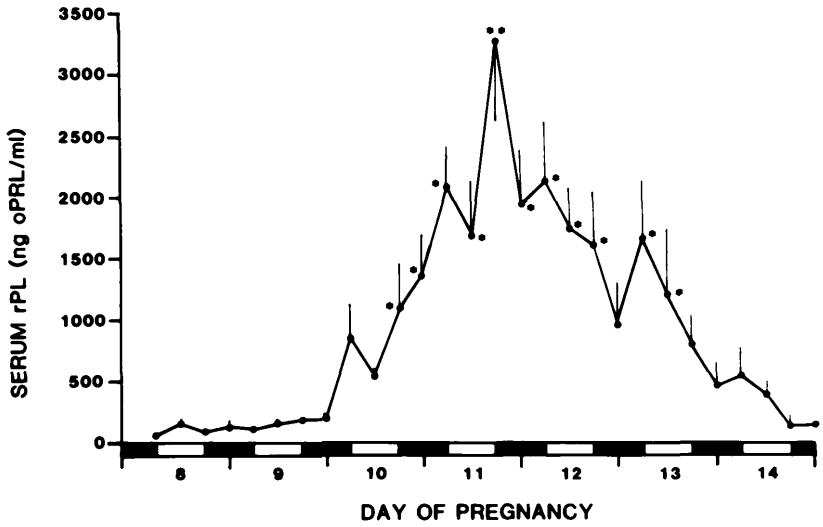

FIG. 3. Serum rPL levels every 6 hr on Days 8 through 14 of pregnancy, as measured by the Nb_2 lymphoma cell bioassay. The bar above or below each point is the standard error. The average number of sera per point was eight. One asterisk indicates a mean which is significantly different from the baseline values on Day 8. Two asterisks indicate a mean significantly different from all other means.

on Day 11, these differences were not present. This work demonstrates that the peak rPL secretion occurs in the late afternoon on Day 11, the same day that luteotropic and mammotropic activity is highest in pregnant rat serum (1, 6, 7, 8).

Prolactin levels for days 8–14 are shown in Fig. 4. The diurnal surge (1800 hr) was seen in only one of eight rats on Day 8, the last day it is normally observed (4). It is known that the diurnal surges are easily suppressed by stress such as surgery (9). It may be that the laporotomy performed on Day 7 inhibited not only the surge on that day but the last diurnal surge on Day 8 as well. Nocturnal surges (0600 hr) were seen on Days 8–10 as

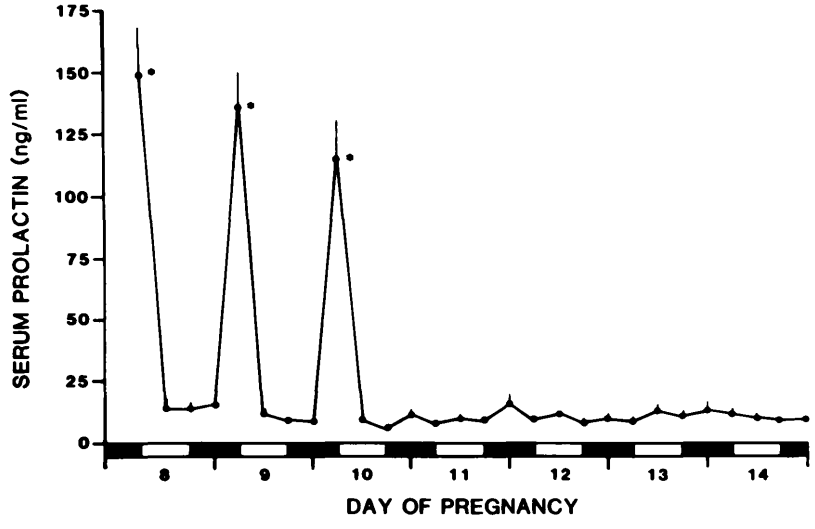

FIG. 4. Serum PRL levels every 6 hr on Days 8 through 14 of pregnancy, as measured by RIA. An asterisk indicates a significant PRL surge.

expected (7). The secretion of the last nocturnal PRL surge is correlated with an increase of rPL levels above baseline on Day 10. This agrees with the inverse correlation between PRL and rPL levels when conceptus number was altered (10). Although rPL levels on this day are about one-third of peak levels 1 day later, they are sufficient to maintain normal development of the conceptus and are sufficient to cause termination of the nocturnal PRL surges (11). It appears that when the luteotropic action of PRL is lost on Day 10 concomitant with the loss of the PRL surges, rPL levels have increased sufficiently to take over as the primary luteotropic factor maintaining the pregnancy.

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Received February 16, 1983. P.S.E.B.M. 1983, Vol. 173.