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# "Micro"-Insulin Radioimmunoassay: Measurement of the Insulin Response during Glucose Tolerance Tests in Guinea Pigs[1] (40783)

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The guinea pig has been utilized as an animal model by numerous investigators in the study of pancreatic islet function (1–5) as well as insulin structure–function relationships (6–11). However, its utility in this regard has been severely limited by the absence of a published procedure which is sensitive and specific for measuring guinea pig insulin in serum or islet incubation media. This situation stems from both the absence of cross-reactivity of guinea pig insulin in other insulin radioimmunoassay systems (12) and the lack of commercial availability of specific antisera against guinea pig insulin.

Studies in our laboratory utilizing the guinea pig as an experimental model required the measurement of low insulin levels in small volumes of serum obtained from multiple bleedings. This paper details a microradioimmunoassay procedure capable of measuring as little as 20 pg of guinea pig insulin. Utilizing this technique, we report for the first time the nature of the insulin response to a glucose tolerance test (GTT) under various feeding regimens in this species.

*Materials and methods.* Purified guinea pig insulin and rabbit anti-guinea pig insulin serum were provided by Dr. C. C. Yip (University of Toronto) and sheep anti-rabbit serum and normal rabbit serum by Dr. L. R. French (University of Wisconsin-Madison). Bovine serum albumin (RIA grade) was purchased from Sigma Chemical Company. Male guinea pigs weighing 300–400 g were purchased from Charles River Laboratories.

*Radioimmunoassay for guinea pig insulin.* Guinea pig insulin was iodinated by the chloramine-T method (13) utilizing $^{125}I$ (Amersham Corp.). The radiolabeled insulin was purified by dialysis (dialysis tubing from Spectrum Medical Industries; exclusion limit 3500 mol wt) against 2 liters of phosphate buffer (0.05 *M*, pH 7.5) with one change for 1 hr to remove unlabeled iodine, followed by purification on a 58 × 0.9-cm column of Sephadex G-50 preequilibrated with 1% bovine serum albumin in 0.05 *M* phosphate buffer (pH 7.5) and eluted with the same buffer without albumin. This procedure yielded a labeled insulin peak with greater than 85% trichloroacetic acid-precipitable radioactivity. The labeled insulin was diluted in 0.05 *M* phosphate buffer (pH 7.5) containing 2% bovine serum albumin (= assay buffer) to contain 2500–3000 cpm per 20-μl volume.

The assay was carried out in 1-ml-capacity microfuge tubes (Fisher Scientific), with all pipetting performed utilizing a 20-μl-capacity Pipetman (Gilson Co.). Standard concentrations of 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.96, 0.98, and 0.49 ng/ml guinea pig insulin in 20 μl volume were run in quadruplicate in each assay. Twenty microliters of the labeled guinea pig insulin solution and 20 μl of a 1:2000 dilution of rabbit anti-guinea pig insulin serum were added to standard and sample tubes, which were mixed on a Vortex mixer and incubated for 48 hr at room temperature. After this time, 20 μl of a 1:10 dilution of sheep anti-rabbit serum containing 1:50 normal rabbit serum was added, and the tube contents were mixed on a Vortex mixer and incubated for an additional 24 hr at room temperature. At the end of the second incubation period, 200 μl of distilled water was added to each tube and the tubes were centrifuged for 10 minutes at 4° and 2500 rpm in

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a Beckman Model TJ-6 centrifuge. The supernatants were aspirated off and the pellets counted in a Packard Auto-Gamma scintillation spectrometer for 10 min (counter efficiency for $^{125}I$ = 78%).

*Glucose tolerance testing (GTT) in fasted and fed guinea pigs.* Twelve guinea pigs were housed under *ad libitum* feeding conditions (Purina guinea pig chow) and then divided into three groups of four animals each. The first group was fasted for 24 hr (fasted group), while the second group was fasted for 24 hr and the food returned for 3 hr (fasted + refed group). The third group was allowed to feed *ad libitum* throughout this period (fed group). At the end of this time, a blood sample (0.1–0.5 ml) was collected from the toenail bed of each animal according to the method of Vallejo-Freire (14). The animals were then injected intraperitoneally with 2 g/kg body weight D-glucose prepared as a 20% solution in distilled water, and bled at 45-min intervals until 3 hr after injection (five samples per animal). At each sampling, the blood was collected into heparinized capillary tubes and expelled into 1-ml-capacity polystyrene microfuge tubes. Following centrifugation of samples at 2500 rpm for 10 min, the serum was removed and a 20-μl aliquot taken for assay of its glucose content by means of the Beckman glucose analyzer. The remainder of the serum was stored at −20° for radioimmunoassay of its insulin content. Each serum sample was assayed in duplicate at two serum dilutions so that parallelism of the sample dilution curve and the dilution curve for guinea pig insulin standard could be assessed. Data from sample dilutions which did not exhibit parallelism to the standard curve, indicating the presence of non-insulin-like substances which interfered with the assay, were not utilized in the calculation of the experimental results.

The data were analyzed by a split plot in time model of the analysis of variance (15), with temporal changes in insulin and glucose levels being determined by trends analysis (16). The latter method was selected since it most accurately assesses the differences in the shapes of the curves, taking into account all of the available data points.

*Results. Radioimmunoassay for guinea pig insulin.* The standard curve for the assay is presented in Fig. 1 as the regression line for the logit transformation. The reproducibility of the system was tested by assaying two dilutions of a pool of normal guinea pig serum in triplicate in each assay. As indicated in Fig. 1, the within-assay and between-assay coefficients of variability, calculated by the method of Rodbard (17), were reasonably small and allowed for reproducible measurements of guinea pig serum insulin content.

The assay was validated for measuring guinea pig insulin in serum by means of both parallelism and recovery trials. As can be seen in Table I, in each of five separate trials, the slopes of the logit regression lines for serial dilutions of normal guinea pig serum were not significantly different from those of the standard curve run in the same assay. As indicated in Table II, the mean recovery of guinea pig insulin standard added to serum and measured in the assay was not significantly different from 100%.

*Glucose and insulin responses to a GTT.* The glucose tolerance curves for the three treatment groups are shown in Fig. 2. There

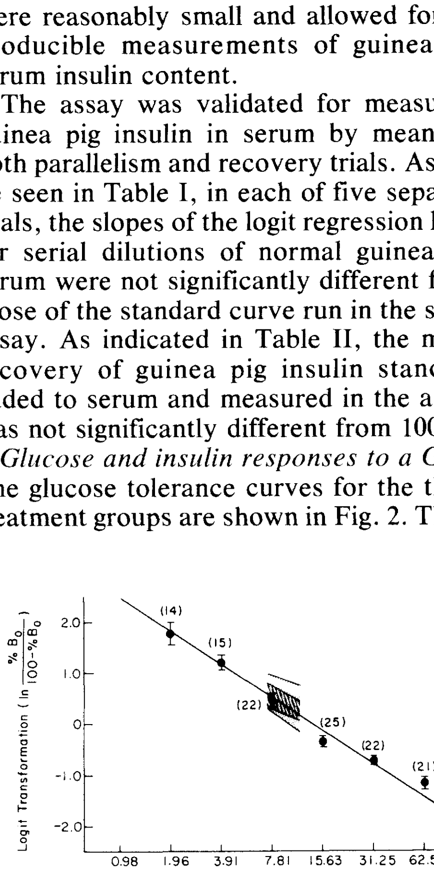


FIG. 1. Standard curve for the logit transformation vs guinea pig insulin concentration. The data were pooled from seven standard curves run in quadruplicate, with means displayed along with twice the standard error corresponding to each length of line above and below; sample sizes are noted within parentheses. $\% B_0$ = counts in tubes containing guinea pig insulin standard × 100/counts in tubes with buffer only. The shaded and cross-hatched areas around the logit least-squares regression line correspond to twice the standard deviation for between- and within-assay variability, respectively, calculated by the method of Rodbard (17) from repeated measurements of pooled serum samples with mean insulin concentrations of 11.21 and 7.48 ng/ml.

TABLE I. RESULTS OF THREE TRIALS OF PARALLELISM BETWEEN GUINEA PIG INSULIN STANDARD AND SERUM DILUTION DOSE–RESPONSE CURVES

| Trial No. | No. of twofold serum dilutions | Slope of logit line for serum dilutions | Slope of guinea pig insulin standard logit line | Student's $t$ |
|---|---|---|---|---|
| 1 | 3 | −0.3750 | −0.5834 | 0.643 |
| 2 | 3 | −0.5423 | −0.6358 | 0.787 |
| 3 | 4 | −0.4929 | −0.5799 | 1.006 |

was a significant ($P < 0.05$) decline in basal (time = 0) blood glucose in the fasted group and a significant increase in the fasted + refed group ($P < 0.01$) as compared to animals fed *ad libitum*. There was a significant difference in the shape of the glucose tolerance curve between the fasted and *ad lib* fed animals (linear trend = $P < 0.01$, quadratic trend: $P < 0.05$) indicative of a higher peak value and slower return to baseline in the former group. The shape of the curve in the fasted + refed animals was not significantly different from either of the other two groups. Nevertheless, some impairment of glucose tolerance is apparent with 90-, 135-, and 180-min glucose levels being elevated as compared to the *ad lib* fed animals.

The insulin responses during the GTT are displayed in Fig. 3. The mean preinjection insulin level in the *ad lib* fed group was 43 ng/ml and was similar in fasted + refed animals. The fasted group exhibited a significant ($P < 0.05$) decline in basal insulin levels as compared to either of the other two groups. In addition, although the *ad lib* fed and fasted + refed groups did not differ significantly from each other in the shape of the insulin response curve, their curves were both significantly different from the shape of the curve for the fasted animals ($P \ll 0.01$ in linear trend for fed vs fasted; $P \ll 0.01$ in cubic trend for fasted vs either fed or fasted + refed).

*Discussion.* Zimmerman (12) has described a radioimmunoassay which can measure guinea pig insulin concentrations as low as 0.4 ng/ml. However, the sample volume required was 10 times greater than that of the present assay and therefore the overall sensitivity was less than that of our procedure. Furthermore, Zimmerman's assay could measure guinea pig insulin across only a 10-fold range of concentrations, whereas our assay is capable of measuring insulin over a greater than 60-fold concentration range. Parallelism and recovery studies were not shown, nor were intra- and interassay variabilities reported, and therefore it is difficult to evaluate the sensitivity and reproducibility of his assays. The parallelism and recovery studies reported here indicate that our assay is a valid technique for measuring guinea pig insulin in small volumes of serum, such as those collected during a GTT.

The effects of fasting on blood glucose levels in the guinea pig are controversial, with some investigators reporting no decline in blood glucose after a 24-hr fast (5, 18), while others have found a decline similar in magnitude to that reported for other mammalian species (12). Although

TABLE II. RECOVERY OF GUINEA PIG INSULIN ADDED TO SERUM AND MEASURED BY RADIOIMMUNOASSAY

| Trial No. | Sample | Insulin (ng/ml) | Percentage recovered, mean ± SE ($n$) |
|---|---|---|---|
| 1 | 1:8 diluted serum | 7.81 | |
| 1 | 1:8 diluted serum + 3.91 ng/ml | 11.47 | 97.49 ± 7.45 (3) |
| 2 | 1:8 diluted serum | 7.11 | |
| 2 | 1:8 diluted serum + 1.96 ng/ml | 12.09 | 133.30 ± 0.55 (2) |
| 3 | 1:16 diluted serum | 3.73 | |
| 3 | 1:16 diluted serum + 15.63 ng/ml | 20.21 | 104.16 ± 14.18 (5)<br>107.99 ± 8.20 (10)[a] |

[a] Pooled from the three trials.

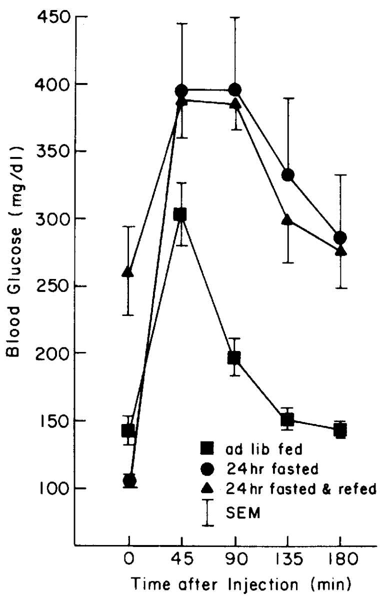


FIG. 2. Blood glucose values following ip glucose injection for fed, fasted, and fasted + refed guinea pigs. Means ($n = 4$ at each point) and standard errors are plotted, with the overall standard error of the mean (SEM) computed from the analysis of variance as SEM = (mean square error/$n$)$^{1/2}$.

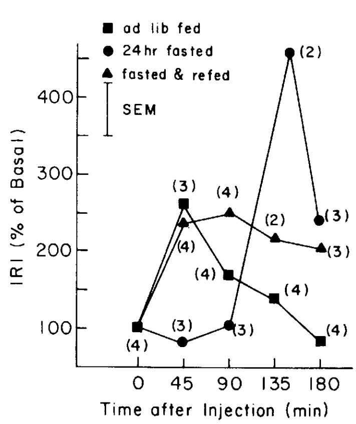


FIG. 3. Serum immunoreactive insulin (IRI) values following ip glucose injection for fed, fasted, and fasted + refed guinea pigs. Means are expressed as percentages of basal ($t = 0$) values to compensate for the high variability in basal values within the groups. Numbers of observations are noted in parentheses, and the overall standard error of the mean (SEM) is depicted, which was computed from the analysis of variance as SEM = (mean square error/harmonic mean of numbers of observations per group)$^{1/2}$. The basal values for the three groups were as follows: *ad lib* fed = 43.01 ng/ml; fasted = 29.24 ng/ml; fasted + refed = 44.33 ng/ml.

previous reports have indicated that blood glucose levels in the guinea pig are highly variable (12, 18, 19), we have not observed such large fluctuations in either fasting or nonfasting glucose levels, and were able to demonstrate a significant decline in blood glucose with a 24-hr fast which was reversed by a 3-hr refeeding period.

Similarly disparate results have been obtained with respect to GTTs in this species, with some investigators reporting an impaired glucose tolerance in apparently normal animals (12, 19) whereas others have found normal GTT curves (18, 20). As far as we are aware, the only reported serum insulin values during a GTT are those of Zimmerman (12). The glucose challenge in this case was administered intraperitoneally after a 24-hr fast, and there was little correlation between blood glucose and serum insulin values, although the latter did display a gradual rise above basal levels during the first 3 hr after the challenge. Our findings in fasted guinea pigs are similar to those of Zimmerman in demonstrating a delayed insulin peak and lack of correlation between insulin and glucose levels during a GTT, although this situation was not present in animals fed *ad libitum* and was normalized by refeeding fasted animals prior to the test. The abnormal insulin responses reported here for fasted guinea pigs and their reversal by feeding are similar to those described following a prolonged fast in other species, in terms of both insulin levels *in vivo* and islet insulin secretion *in vitro* (21–23). However, since the glucose tolerance in the fasted + refed group remained somewhat impaired, the insulin resistance reported during fasting in other mammalian species (24, 25) may also be present in the guinea pig and require more than 3 hr of feeding to be fully reversed.

Of particular interest is our confirmation of a previous report (6, 12) that basal insulin levels in the guinea pig, although highly variable, are considerably greater than those described for other mammalian species. The mean serum insulin concentration in our animals fed *ad libitum* was 43 ng/ml, whereas generally accepted serum insulin levels in man and rat are less than

10% of this value. The specific cause for this relative hyperinsulinemia in the guinea pig remains to be elucidated, but may represent a compensatory adjustment for the low biological activity of guinea pig insulin (6). Further studies are needed to determine whether hyperinsulinemia in this species represents true hypersecretion or, alternatively, a decrease in the metabolic clearance rate of guinea pig insulin.

*Summary.* A method is presented for the radioimmunoassay of insulin levels in small volumes of serum collected by multiple bleedings of guinea pigs. The validity of this technique was evaluated by both parallelism and recovery studies. The assay was utilized to measure the insulin response to a glucose tolerance test in fed, fasted, and fasted + refed groups of animals.

Insulin levels in guinea pigs under basal conditions were 10-fold higher than those reported for other species. A significant decline in both basal blood glucose and insulin levels occurred following a 24-hr fast. Fasted guinea pigs also demonstrated an impairment of glucose tolerance and a delayed insulin peak; both of these abnormalities were reversed by refeeding. These results suggest that although the guinea pig possesses an unusually high blood level of immunoreactive insulin, its insulin and glucose responses to fasting and glucose tolerance tests are similar to those reported for other species.

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