Development and Application of a Radioimmunoassay for Plasma Glucagon

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We report sensitive radioimmunoassay procedures for glucagon-like immunoreactivity and for pancreatic glucagon, with use of antisera generated in rabbits by the injection of glucagon covalently coupled to thyroglobulin. Optimum assay conditions were determined. These assays have absolute limits of sensitivity of 10 to 20 pg, an intra-assay coefficient of variation of 8%, and an inter-assay coefficient of variation of up to 22%. In human infants 30 minutes after birth, glucagon concentration was 227 ± 27 pg/ml (SEM), and glucagon-like immunoreactivity increased sharply with oral feeding. In children and adolescents, plasma glucagon concentrations during fasting, the increase in response to arginine stimulation, and the suppression after oral glucose loading were similar to values previously reported in adults. Problems inherent in the methodology for measuring plasma glucagon are discussed.

Additional Keyphrases: pancreatic glucagon and glucagon-like immunoreactivity in plasma • values in newborns, children, adolescents, adults • stimulation and suppression of values • specificity of antisera • hormones

The significance of pancreatic glucagon in glucose homeostasis in health and disease is becoming increasingly more apparent, largely because radioimmunoassay has been used to measure glucagon in plasma (1, 2). Several such reports have appeared (3-7), but a critical review (8) highlights several problems and indicates that, with few exceptions, the assays have not adequately distinguished between pancreatic glucagon and glucagon-like materials derived from the gut and perhaps other sources. The terms "enteroglucagon" and "glucagon-like immunoreactivity" have been applied to these cross-reacting materials. There are some reports regarding measurement of plasma glucagon by use of radioimmunoassays highly specific for pancreatic glucagon (8-11); however, most recent investigations report data on plasma pancreatic glucagon concentrations measured by use of specific antisera obtained from either Heding or Unger et al. (8, 12-18). Thus, a variety of specific glucagon antisera have not been readily available, and quality-control data regarding glucagon measurements have been limited. This paper describes the development of radioimmunoassay systems for glucagon-like immunoreactivity and for pancreatic glucagon, and their preliminary application to the investigation of the role of glucagon in the human newborn and in children.

Materials and Methods
Antibody Generation
Antisera to pancreatic glucagon were generated in New Zealand White female rabbits by repeated subcutaneous injection of glucagon covalently coupled to bovine thyroglobulin via the carbodiimide reaction (19). The rationale for, and successful use of, thyroglobulin to produce antigenicity to small molecules has been previously described (19).

The extent of coupling was assessed by adding radiolabeled glucagon to the reaction mixture and extensively dialyzing the reacted material against water. Molar ratios of glucagon to thyroglobulin to carbodiimide in the reaction mixture of 100:1:200 consistently resulted in incorporation of up to 30 moles of glucagon per mole of thyroglobulin. The conjugate was emulsified in complete Freund's adjuvant, and 0.5 to 1.0 mg of glucagon (1 ml of emulsion) was injected at two- to four-week intervals for as long as a year. Conjugated, highly purified, beef-pork crystalline glucagon (a generous gift of Dr. Mary Root, Eli Lilly and Co., Indianapolis, Ind.; Lot No. 258-234B-167-1) was initially injected; conjugated partially purified glucagon (Calbiochem, San Diego, Calif. 92112; Lot No. 100-828) was used for booster injections. The animals were bled monthly, and serial dilutions of serum were checked for their ability to bind 125I-labeled, highly purified, crystalline glucagon. Selected antisera also were screened for reactivity with insulin, gastrin, and an extract of crude pork-gut glucagon-like material (CGRP) (kindly supplied by Dr. Lise Heding, Novo Institute, Denmark). This material has an estimated potency of 2000 ng equivalents of glucagon-like immunoreactivity per milligram of powder (Dr. Heding, personal communication). Finally, the specificity and sensitivity of our most potent antiserum was compared to that of antiserum 30K of Unger et al.; this latter antiserum now is widely used and is reported to be highly specific for pancreatic glucagon (14-17).

Stock Solutions and Standards
Buffer: The buffer used to dilute all antisera and standards was phosphate-buffered saline (10 mmol of phosphate and 9 g of NaCl per liter, pH 7.4) con-
taining 2.5 ml of normal rabbit serum per liter.

Standard glucagon solutions: 200–500 µg of purified (Lilly) glucagon was dissolved in HCl (20 mmol/liter) to an initial dilution of 200 µg/ml. Serial dilutions were then prepared with the buffer to yield solutions in which 100 µl contained 2, 5, 10, 20, 30, 40, 50, 100, 200, 500, or 1000 µg of glucagon. These stock solutions were divided into small aliquots and frozen; each aliquot was used once only and then discarded. Fresh standards were prepared every eight weeks, because there was some loss of potency of the standard solution after this time. The initial preparation also was used for iodination.

Disodium ethylenediaminetetraacetic acid (EDTA), pH 7.4.

Aprotinin (Trasylol; FBA Pharmaceuticals, New York) was used at a final concentration of 500 kallikrein inhibitor units (50 µl) per milliliter for the collection of blood samples and in the assay procedure (5–7). Its function is to minimize glucagon degradation.

Antisera were diluted with the protein-containing buffer to the working dilution. Final dilutions in the radioimmunoassay systems range from 50,000 to 300,000-fold.

Low-glucagon plasma was obtained from a pancreactomized dog.

Glucagon iodination

Crystalline glucagon was iodinated by a modification of the chloramine-T method of Greenwood et al. (20). Two to four micrograms of glucagon was reacted with 2 mCi of 125I in the presence of 10 µg of chloramine-T for 15–30 s; the reaction was then stopped by adding 10 µg of sodium metabisulfite. Iodinated hormone was separated from inorganic iodide on a disposable 0.5 × 20 cm column of Sephadex G-10. The column was equilibrated with the phosphate-buffered saline and washed with a solution of bovine serum albumin (20 g/liter). The same buffer was used for elution; 0.5-ml aliquots were collected, and portions from the protein peak were tested for their ability to bind with glucagon antibody (10,000-fold dilution). Only material reacting to the extent of 80% or more was used; the specific activity usually exceeded 400 Ci/g. A highly purified moniodinated glucagon could be separated from di-iodinated or damaged glucagon by separation on an anion-exchange column (QAE Sephadex A25, 0.5 × 30 cm) as recently described (21). 125I-Labeled glucagon (Cambridge Nuclear Corp.) was consistently of good quality. Any of these three labeled glucagon preparations which met the criteria of binding outlined above proved to be satisfactory in our radioimmunoassay systems.

Radioimmunoassay Method

Standard curves in buffer were constructed by reacting duplicate 100-µl aliquots of standard glucagon (5000 pg) with 100 µl of antiserum (50,000 to 300,000-fold final dilution) in a total volume of 1 ml. The reaction mixture also contained 100 µl of the EDTA, 50 µl of Trasylol, and 100 µl of 125I-labeled glucagon (20 pg). Control tubes without standard (“100% tubes”) and control tubes without antiserum (“blank” or “nonspecific binding” tubes) were set up with each standard curve. Pre-incubation for 48 h before the delayed addition of the radiolabeled glucagon enhanced the sensitivity of the radioimmunoassay. Subsequently, the incubation was allowed to proceed for a further 72 h. Free and bound 125I-labeled glucagon were separated by using a predetermined optimum amount of goat anti-rabbit globulin antiserum (second antibody). The second-antibody reaction proceeded 48 h. All incubations and additions were done at 4 °C. When plasma samples were to be assayed the standard curve was controlled with low-glucagon plasma. The volume of plasma in the tubes for assay and in those for the standard curve did not exceed 200 µl. Curves for buffer and plasma were parallel when all tubes contained an identical amount of protein. Counting was done with an automatic gamma scintillation counter, and results were calculated as previously described (22). Absolute percentage bound was determined by counting the tubes before and after separation of supernate, and after subtracting values for blank tubes. Depending on the number of samples being assayed, total counts were determined either in all tubes or in every tenth tube.

Sensitivity and intra-assay variability were determined in an assay with 10 replicates at each point. Inter-assay variability was determined by repetitive measurements of a pool of plasma from fasting subjects and of a pool of plasma obtained from a normal subject undergoing an arginine stimulation test. Recovery of known amounts of glucagon added to plasma was estimated on several occasions. Blood samples were collected into chilled tubes containing EDTA and Trasylol. The plasma was separated by centrifugation as soon as possible and stored at −20 °C until assayed.

Results

Validation of the Radioimmunoassay Systems

Cross-reactivity. In each of seven rabbits immunized over a period of one year, antisera were produced that at final dilutions of 10,000-fold or greater could bind 50% or more of a tracer amount (20 pg) of 125I-labeled glucagon. Two antisera, Gl-1 and Gl-5 are currently being used. Both will bind up to 30% of a tracer dose of 125I-labeled glucagon (20 pg) in final dilutions of 200,000 to 300,000-fold. Representative standard curves of Gl-1 and Gl-5 are shown in Figures 1 and 2. Figure 1 shows glucagon and CPGG reactions for the Gl-1 antiserum. The CPGG and glucagon curves are not parallel, so that exact potency comparisons are not possible, but in terms of weight the cross-reaction of CPGG is in the range of 1000–2500 to 1 (2.5 to 1 glucagon equivalents). In
contrast, antiserum GI-5 is more specific for pancreatic glucagon (Figure 2); the extent of CPGG cross-reaction approximates 50,000:1 at the midportion of the nearly parallel curve (100:1 glucagon equivalents). The cross-reactivity of the CPGG with Unger’s 30K antiserum is nearly identical. Neither GI-1 nor GI-5 showed significant cross reaction with insulin or gastrin. Plasma containing a high concentration of endogenous pancreatic glucagon gave a dilution curve parallel to the standard curve (Figure 3), further suggesting specificity.

Sensitivity. Extensive studies were conducted to assess the factors necessary to guarantee high sensitivity of the radioimmunoassay system. Delayed addition of tracer increased sensitivity. A 24-h pre-incubation followed by an additional 48 h yielded satisfactory curves, but 48 h of pre-incubation and 72 h final incubation increased the sensitivity to 10-20 pg glucagon per tube. The addition of protein (serum or plasma) significantly depressed the absolute binding of 125I-labeled glucagon to antibody, and this effect was more marked with high concentrations of protein. This did not appear to be attributable to cross-reacting materials, because we observed similar displacement of the absolute binding for plasma from pancreatectomized dogs, and after addition of bovine serum albumin (50 g/liter). A similar phenomenon has recently been described for Unger’s antiserum 30K (23). However, curves obtained by preparing the assay with buffer alone or with buffer containing plasma from a pancreatectomized dog were superimposable when plotted as B/B₀ > 100, where B equals counts bound for each standard, and B₀ the counts bound in the absence of cold antigen. Unknown sample was always assayed by reference to a standard curve which, throughout incubation, contained an equivalent volume of low-glucagon plasma.

Sensitivity was markedly affected by the quality of the 125I-labeled glucagon, and was optimal using monoiodinated material that bound to the extent of 80% or more to a 10,000-fold dilution of antibody GI-5. Under these conditions, nonspecific binding (in blank tubes) was usually less than 5%, and did not exceed 7% of total counts, in buffer or plasma curves. Thus, this nonspecific binding reflected “damaged” labeled hormone.

For antiserum GI-1 at a final dilution of 200,000-fold, the absolute sensitivity of the radioimmunoassay system, determined from a curve containing 10 replicates at each point, was 20 pg. This point represents the amount of glucagon distinguishable from values for the 100% tubes at the 95% confidence level. The intra-assay coefficient of variation did not exceed 8% at any point and was 4% in the midrange of the curve. With GI-5 antiserum at a final dilution of 300,000-fold and 10 replicates at each point, the absolute sensitivity of the radioimmunoassay system was 10 pg. The intra-assay coefficient of variation ranged from 1.7 to 7.6% and was 3.4% in the midrange of the curve.

Inter-assay variability was determined by using aliquots of a pool of plasma from a patient undergoing an arginine stimulation test after an overnight fast. This was estimated on seven separate occasions with antiserum GI-1. The mean estimate was 583 pg/ml (range, 400-750 pg/ml) and the standard error of the mean (SEM) was 29 pg/ml. Thus, the inter-assay coefficient of variation was 13.2%. The same pool assayed with antiserum GI-5 on six separate occasions gave a mean estimate of 450 ± 39 pg/ml (range, 300-660 pg/ml) and an inter-assay coefficient of variation of 22.9%.
Recovery studies were performed by adding crystalline glucagon to plasma samples containing about 100 pg of endogenous glucagon per milliliter. When 500, 1500, or 1500 pg/ml was added on three separate occasions, the mean recovery, with use of GI-5 antiserum, was 117%, 103%, and 111%, respectively.

The effects of omitting Trasylol in the collection of blood specimens or in the assay incubations was not critically examined because of the firm evidence for its protective role in preventing glucagon degradation (5, 7, 29).

Application of the Radioimmunoassay Systems

We have used the present assay systems to measure glucagon immunoreactivity in the plasma of newborn infants and children.

An example of the differences in results obtained when antisera GI-1 and GI-5 were used simultaneously is seen in Table 1. We compared 10 plasma samples obtained from healthy newborn infants at various times after birth and (or) feeding. With GI-1 it can be seen that values for glucagon immunoreactivity were consistently much higher than the values for pancreatic glucagon (GI-5 immunoreactivity). However, there was no direct relationship between values obtained by the separate antisera, and the coefficient of correlation was only 0.27.

The infants were fed glucose solution at 12 h and formula at 24 h. Plasma glucagon-like immunoreactivity increased with age, presumably in response to feeding, but the increase in pancreatic glucagon was less marked. In 17 healthy newborns, plasma glucagon-like immunoreactivity (GI-1 antiserum) 30 min after birth and before the first oral feeding was 227 ± 27 pg/ml. Glucagon immunoreactivity was maximal at 72–96 h of age in infants fed formula beginning at 24 h. Thus, plasma glucagon concentrations in the fasting newborn are similar to those reported for fasting adults (1), and glucagon-like immunoreactiv-

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<th>Table 1. Total Immunoreactive Glucagon and Pancreatic Glucagon Concentrations in Plasma of 10 Normal Human Newborns*</th>
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Coefficient of correlation = 0.27

* Infants delivered normally without complications. Neonatal course uneventful.

In four healthy adolescents who had fasted overnight, the intravenous infusion of not more than 30 g of arginine (0.5 g/kg body wt) during 30 min increased the mean (±SEM) plasma glucagon concentration from a pre-infusion value of 42 ± 7 pg/ml to a peak of 341 ± 45 pg/ml at 30 minutes; by 1 h, values had declined to 143 ± 45 pg/ml. The plasma pancreatic glucagon concentration (GI-5 immunoreactivity) in 10 fasting healthy non-obese children was 131 ± 31 pg/ml. Plasma glucagon concentrations declined from a basal value of 122 ± 25 pg/ml to 92 ± 14 pg/ml at the second hour of an oral glucose-tolerance test in seven children (P <0.05 by paired t analysis). Thus, in children, plasma glucagon concentrations in the basal state, after stimulation with arginine or suppression with glucose, are similar to values previously reported in adults (1).

Discussion

The problem of generating antisera of adequate sensitivity against small polypeptide antigens has been overcome in several laboratories by the use of antigen that has been covalently coupled to some one of a variety of larger molecules, or by forming antigen polymers (3, 8, 11, 24). In our laboratory, thyroglobulin has been successfully used as the ligand in development of high-affinity antibodies against triiodothyronine, thyroxine, vasopressin, oxytocin, and parathyroid hormone, as well as glucagon (19). However, this approach does not guarantee antibody specificity, and for glucagon the problem of specificity—i.e., the ability of the antibody to discriminate between pancreatic glucagon and glucagon-like immunoreactive materials derived from the gastrointestinal tract—is difficult to overcome. Most glucagon antisera cross-react to varying degrees with intestinal extracts. There is small likelihood of producing a relatively specific antibody, such as the GI-5 antiserum we used here, by this conjugation technique. Generating antisera to various segments of the glucagon polypeptide chain is more likely to produce specific antisera (25, 26).

There are several other problems that complicate radioimmunoassay of many polypeptide hormones, including glucagon, in plasma. The tendency to rapid enzymatic degradation of native endogenous glucagon in plasma or of radiolabeled glucagon can be overcome by the use of Trasylol; more recently-Benzamidine has been proposed as an alternative, but we have not explored its use (27). The loss of potency of stored standards, which in the case of glucagon necessitates preparing fresh standards about every two months, introduces a further error. Also, it is difficult to produce labeled hormone of high specific activity that is stable and binds reliably to antibody. Purification of newly labeled hormone by chromatography on a Sephadex anion-exchange column appears to be superior to
simple gel filtration, in that binding of mono-iodinated glucagon to antibody is enhanced (21). Finally, the critical effects of temperature and high protein concentrations have been demonstrated (8). These problems have combined to make radioimmunoassay of glucagon particularly difficult; quality control data are needed before results are published.

The present radioimmunoassay systems fulfill the criteria of high sensitivity and specificity for pancreatic glucagon. With antisera GI-5, results are comparable to those obtained with antisera 30K (Unger); standard curves are nearly superimposable and measured plasma glucagon concentrations in the fasting or stimulated state are similar to results reported by others using antisera 30K (1, 3, 14, 17). In addition, the suppression of plasma glucagon concentration during an oral glucose-tolerance test further attests to the specificity of the GI-5 antisera for pancreatic glucagon. Replicate measurements in the radioimmunoassay systems indicate that intra- and inter-assay variation is acceptably small. The parallelism of the standard curve and the curve obtained for serial dilutions of plasma further validates the present assay systems. We have used the assays in the sheep fetus (28), and our preliminary results attest to the applicability of our systems.

The concomitant use of antisera that discriminate (GI-5) and fail to discriminate (GI-1) between glucagon and glucagon-like material makes it possible to measure concentrations of pancreatic glucagon and glucagon-like immunoreactivity in plasma simultaneously, and to assess the contribution of pancreatic glucagon to glucagon-like immunoreactivity. In the fasting state the differences for the two in pancreatic glucagon will not be great, because glucagon-like immunoreactivity of enteric origin is not present in high concentrations. This is borne out by the similarity of the glucagon concentrations after arginine infusion, whichever antisera is used to make the measurement. In contrast, when both antisera GI-1 and GI-5 were used to measure glucagon immunoreactivity in 10 infants at various times after feeding and up to 5 days of age, most of the circulating glucagon-like immunoreactivity was clearly of nonpancreatic origin.

Total glucagon-like immunoreactivity was greatest in infants 3–5 days old, when glucose concentrations have returned to euglycemic values; qualitatively similar but smaller changes in pancreatic glucagon were also apparent. The overall significance of glucagon secretion in the neonatal period is yet to be determined.

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References