Interference of Immunoglobulins in Two Glucagon Radioimmunoassays

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Radioimmunoassays of glucagon in plasma may be complicated by interaction with other substances of high molecular mass. Precipitates of such substances with ammonium sulfate showed, after isoelectric focusing, two fractions having glucagon immunoreactivity. One fraction (pI ~10) evidently is associated with the Fc portion (but not the Fab portion) of purified polyclonal immunoglobulin G (IgG). Equal amounts of purified monovalent IgG of various subclasses, especially IgG 1, gave different "glucagon" readings, suggesting that some IgG may interfere more strongly than others. The other fraction (pI 5–6) appeared less consistently, and on gel chromatography appeared to be slightly larger than IgG. Together these fractions add about 50–100 ng/L to the immunoreactive glucagon values in plasma. Therefore methods in which glucagon is extracted before assay should be used for determining the concentration of glucagon present physiologically.

Additional Keyphrases: variation of variation of isoelectric focusing cross-reacting polypeptides monoclonal and polyclonal immunoglobulins

Radioimmunoassay of pancreatic glucagon requires antisera directed against the carboxyl-terminal region of the glucagon molecule, to avoid concurrent determination of the glucagon-like immunoreactive polypeptides from the gut (1). Measurements of glucagon in plasma by carboxyl-terminal directed antisera such as Unger's "antisem 30 K" (1), however, may include precursor molecules (2) and substances of high relative molecular mass (high-M₆) (3) described in 1974 by Valverde et al. as "big plasma glucagon" (3). Neither the identity nor source of these high-M₆ substances has yet been clarified.

According to the literature, "big plasma glucagon" appears to be associated with immunoglobulin G (IgG) in many ways (3), but no correlation between the concentrations of IgG and "big plasma glucagon" in plasma was found when "big plasma glucagon" was measured with antisem 30 K (4). We have recently prepared a glucagon antisem, E7, that recognizes the same immunoreactive components in plasma as does antisem 30 K (5, 6). However, antisem E7 measured zero values for high-M₆ substances in a patient with agammaglobulinemia, and gave higher values for these high-M₆ substances than does antisem 30K (5). Therefore, we used antisem E7 to reinvestigate the relation between these substances and IgG. Our study shows that preparations of the high-M₆ substances from human plasma are heterogeneous on isoelectric focusing. The results suggest that IgG is one of the sources of the high-M₆ substances. The results demonstrate as well the presence in plasma of another fraction of high-M₆, which needs further characterization.

Materials and Methods

Preparation of the high-M₆ substances from plasma: We precipitated 100–200 mL aliquots of citrated plasma from six blood donors by adding solid ammonium sulfate (final concentration, 1.9 mol/L) and allowing the mixture to stand for 2 h at 4 °C. The precipitate, obtained by centrifugation at 400 x g, was dialyzed against ammonium bicarbonate solution (10 mmol/L, pH 8) containing 1 mmol of NaN₃ per liter. The dialysate was then subjected to high-pressure ultrafiltration (Diaflo membrane XM 100A; Amicon, Ousterhout, Holland). One volume of de-ionized water was added, and when the contents of the ultrafiltration chamber had reached 50 mL, they were centrifuged and subjected to isoelectric focusing.

Isoelectric focusing: For preparative electrofocusing at 4 °C we used a sucrose density gradient with an ammonylate range of pH 3.0–10.0 broadened between pH 5–7 (Ampholines; LKB, Bromma, Sweden) on a 110 mL LKB 8100 column; the LKB 2103 power supply was used according to the manufacturer. Aliquots of 50 to 100 mL of preparations of the high-M₆ substances were applied. In other experiments, 100 mg of purified IgG was applied. The pH of the column effluent was checked at room temperature and corrected to 4 °C (7). The IgG concentrations (8) and the interference in the glucagon radioimmunoassays—expressed as immunoreactivity equivalent to glucagon (see below)—were measured in all the fractions without corrections in the standard curves for sucrose or ammonylate content. To control the molecular size of the focused substances, we chromatographed fractions pooled according to the glucagon-equivalency profile on a 1.5 x 60 cm column of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) in assay buffer (see below) and reasayed for immunoreactivity equivalent to glucagon.

Preparation of immunoglobulins: To substantiate the interference of IgG in the glucagon radioimmunoassay, we purified polyclonal IgG from a pool of sera from several hundred registered blood donors by two methods (9, 10). Part of the polyclonal IgG prepared by anion-exchange chromatography (10) was further purified by gel chromatography on Sephadex G-200 (Pharmacia Fine Chemicals).

Monoclonal human immunoglobulins of the D, M, and G subclasses were prepared from plasma of patients with multiple myeloma or Waldenström's macroglobulinemia. A combination of ammonium sulfate precipitation (11), ion-exchange and gel chromatography (10), and immunoadsorption (12) was used to purify the monoclonal immunoglobulins. Their purity was checked by analytical methods (13–16) and was always found to exceed 90% by these criteria. The subclass of the monoclonal IgGs was determined as described elsewhere (17, 18). All preparations of polyclonal IgG and monoclonal immunoglobulins were dialyzed exhaustively against distilled water and lyophilized before being stored at room temperature.

To exclude contamination of the immunoglobulins by glucagon, aliquots of the 20 g/L polyclonal IgG solution in assay buffer were subjected to gel filtration on Sephadex G-200. All immunoreactivity equivalent to glucagon was found in the IgG peak, none in the portion of the effluent in which glucagon appears.

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Trying further to localize a possible binding area for glucagon to the different parts of IgG, or a cross-reacting structure in the immunoglobulins caused by a "glucagon-similar" sequence, we prepared the fragments containing the antigen-binding portions (Fab) and the constant portions (Fc) of the polyclonal IgG by mercuri-papain digestion essentially as described by Hsiao and Putnam (19), except that the IgG was incubated for only 30 min before the digestion was terminated by addition of recrystallized iodoacetic acid. The Fab and Fc fragments were separated from each other by preparative agarose gel electrophoresis (13), then freed of small amounts of undigested IgG and desalted by gel filtration on a column of Ultrogel AcA 54 (LKB) and finally lyophilized.

**Determination of immunoreactive glucagon (IRG):** Porcine glucagon (highly purified), from Novo AS, Copenhagen, Denmark) was used as standard and for iodination (20). Our own antiserum E7 (5) and antiserum 30K (from the Diabetes Research Fund, southwestern Medical School, Dallas, TX), each of which recognizes a part of the carboxyl-terminal region of glucagon, have previously been characterized (5). Both antisera measure high-M_r substances with immunoreactivity equivalent to glucagon in the same fractions of eluate when plasma is separated on a Sephadex G-200 column (5). We used antiserum E7 in all our measurements. In addition, antiserum 30K was used in the experiments with purified IgG and with plasma stripped from IgG as stated. The assay conditions were those recommended for antiserum 30K (21) but with the normal sheep serum omitted from the assay buffer. The assay buffer was 0.2 mol/L glycine, adjusted to pH 8.8 with sodium hydroxide and containing, per liter, 2.5 g of albumin (human serum albumin, 100% electrophoretic purity; Behringwerke, Marburg, F.R.G.), 0.1 mmol of thimerosal, and 500·10^(-3) kallikrein inhibitor units of aprotinin (TrasyloI; Bayer, Leverkusen, F.R.G.). This buffer was also used in all gel-electrophoresis experiments. Blanks from which antiserum was omitted were included for all samples. The sample volume was 200 µL, the final assay-tube volume 1200 µL. In some instances the sample volume was increased to 500 µL to improve the sensitivity. Dextran-coated charcoal was used to separate bound from free glucagon (5). The detection limit of the glucagon assays was 15 ng/L. All results with both antisera are presented as "immunoreactivity equivalent to glucagon" (IRG).

**Interference of purified immunoglobulins in the glucagon assay:** We measured IRG in the different immunoglobulin preparations several times, in duplicate, using 200 µL of immunoglobulin-antibody buffer solutions containing 0.1 to 100 g of the lyophilized immunoglobulin per liter. The immunoglobulin solutions with concentrations of 50 g/L or greater were turbid, but when 200 µL was added to glucagon assay tubes containing 500 µL of diluted antiserum and 500 µL of 125I-labeled glucagon, the final mixtures were essentially clear. Both antisera E7 and 30K were used for these measurements.

To see whether the fractions containing IgG in the isoelectrically focused plasma were associated with interference, we removed IgG from plasma by treatment with staphylococcal protein A before subjecting the plasma to isoelectric focusing. Protein A—known to bind IgG1, IgG2, and IgG4—was purchased as Protein A—Sepharose CL-4B (Pharmacia Fine Chemicals) and packed in a 5×7 cm column. We divided 100 mL of fresh citrate-treated plasma into two aliquots and dialyzed one 50-mL portion against 50 mmol/L Tris HCl buffer (pH 7.4) containing 0.5 mol of NaCl per liter, then applied it to the protein A column equilibrated in the same buffer mixture. The column eluate was monitored for absorbance at 280 nm, and ultraviolet-absorbing fractions were pooled and concentrated to the original volume by high-pressure ultrafiltration. IgG measurements (8) revealed that 98% of the IgG was removed on its passage through the column. Electrophoresis on agarose gel (13) confirmed that concentrations of the other proteins were unchanged. Both the untreated and IgG-striped plasma were precipitated with ammonium sulfate and subjected to isoelectric focusing.

**Results**

Isoelectric focusing of the high-M_r substances obtained by precipitation of plasma with ammonium sulfate resulted in two recurrent localizations of IRG in six individuals tested. Figure 1 depicts the results of one of these experiments. One IRG profile corresponded with the IgG concentration profile. Gel chromatography on Sephacryl S-200 of the pooled fractions containing the IRG again revealed the correspondence between the immunoreactivity and the IgG elution volume.
This was so in five of six experiments; in one case IRG was undetectable. Uncorrected for the presence of amylolytes and sucrose, estimates of IRG in the column effluents ranged from 0 to 180 ng/L at IgG concentrations of 10 g/L. Isoelectric focusing of polyclonal IgG that had been purified for control experiments by anion-exchange and gel chromatography also revealed an IRG profile that closely conformed to the profile of IgG. In contrast, we found no such immunoreactivity in the isoelectrically focused plasma that had been freed from IgG by treatment with protein A (Figure 2).

In four of six experiments, another IRG was localized in the pH range between 5 and 6 (or close to 5) (Figure 1). This profile was not as consistent in shape and localization as the one seen in the IgG concentration profile. The immunoreactivity amounted to 5 to 55 ng of apparent glucagon per liter of the original plasma. Gel chromatography on Sephacryl S-200 of the pooled fractions from the two experiments with the highest IRG concentration showed that its main portion eluted slightly before the 125I-labeled IgG-marker (Figure 3), suggesting a part of this fraction to be of somewhat greater molecular size. This IRG was not found in the control experiments in which the polyclonal IgG was subjected to isoelectric focusing.

In some cases we saw a third IRG, not exceeding 1 ng/L of the original plasma in the pH range 4-4.4, where the albumin was focused.

Table 1 gives our measurements with antisera E7 and 30K confirming the presence of IRG in the purified immunoglobulins in the absence of sucrose and amylolytes. All of our IgG preparations interfered in the glucagon assays. IgG concentrations of 10 g/L were measured as at least 30 ng of IRG per liter. This also happened with the Fc fragments of purified polyclonal IgG, but not with the Fab fragments. The Fc fragments had the greatest apparent glucagon immunoreactivity per milligram. Figure 4 shows the interference produced by increasing amounts of polyclonal IgG purified by ion-exchange and gel chromatography.

Some, but not all, purified monoclonal immunoglobulins of the IgG subclasses 2–4 (investigated at the concentration of 10 g/L) also displayed a significant IRG, although to a lesser degree than did the polyclonal IgG or its Fc fragments. IRG varied greatly among 11 different monoclonal immunoglobulins of the IgG 1 subclass: equal amounts (10 g/L) showed IRG ranging from 15 to 135 ng/L (mean, 60 ng/L). Monoclonal immunoglobulins of the M and D classes in 1 g/L concentration interfered insignificantly.

**Discussion**

Isoelectric focusing of high-MI, precipitates from plasma containing "big plasma glucagon" (3), a component having IRG and found in human plasma but so far unidentified, demonstrated a heterogeneity of this component. In addition to a high-MI component having IRG and a pI near 5, IRG also was found in fractions corresponding to IgG, which disappeared when IgG was removed from plasma. Assay of in-

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**Table 1. IRG Determination in Purified Immunoglobulins, with Use of Antisera E7 and 30K**

<table>
<thead>
<tr>
<th>Polyclonal IgG purified by:</th>
<th>Weighed amount of Immunoglobulin</th>
<th>Mean concn of immunoreactivity equivalent to glucagon (and SD)</th>
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<tr>
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<td>g/L</td>
<td>μmol/L</td>
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<tr>
<td>Acridine lactate/</td>
<td>10</td>
<td>60</td>
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<tr>
<td>ammonium sulfate (9)</td>
<td>50</td>
<td>300</td>
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<tr>
<td>DEAE-Sephadex chromatogr. (10)</td>
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<td>600</td>
</tr>
<tr>
<td>DEAE-Sephadex and Sephadex G-200 chromatogr. (10)</td>
<td>6.7</td>
<td>40</td>
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<td></td>
<td>10</td>
<td>60</td>
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<td>67</td>
<td>400</td>
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<td>Fc fragments b</td>
<td>10</td>
<td>200</td>
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<tr>
<td>Fab fragments b</td>
<td>10</td>
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* The weighed amounts and the results are given both as mass concentration (g/L) and as substance concentration (mol/L). The normal concentration of polyclonal IgG in human plasma is 10 g/L. The IgG interference is tested in increasing amounts. "Eq" means immunoreactive equivalent amount of apparent glucagon. b From polyclonal IgG purified by chromatography on DEAE-Sephadex and Sephadex G-200.
increasing amounts of differently purified polyclonal IgG showed that IgG interfered in proportion to its concentration in two glucagon assays. Thus, our study confirms previous reports that immunoglobulins interfere with the radioimmunoassay of glucagon (3, 4, 22, 23).

The nature of this interference of IgG in the glucagon radioimmunoassay was further investigated. We could exclude interference caused by contamination of the IgG preparations with free glucagon or glucagon-precursor molecules (2). We used several different methods for purifying the polyclonal IgG, and gel chromatography of the polyclonal IgG on Sephadex G-200 did not reveal any immunoreactivity in the elution volumes corresponding to either glucagon or precursor molecules. Nor could our findings be explained by a high content of small molecules or salt in the polyclonal IgG disturbing the antigen-antibody reaction in the assay, resulting in artificially high glucagon readings. All preparations had been desalted by dialysis or gel chromatography. Fab fragments gave markedly different results in spite of the identity of the procedures used in their purification: preparative agarose electrophoresis and gel chromatography.

The localization of the activity interfering in the glucagon assay to the Fc fragment of the polyclonal IgG but not to the Fab fragment excludes the possible presence of autoantibodies to glucagon in the immunoglobulin preparations. However, some glucagon still could be bound to the Fc portion. Such glucagon might dissociate or exchange with 125I-labeled glucagon under glucagon assay conditions, thus giving rise to difficulties in interpretation of the assay results. Also a "glucagon-similar" sequence present in the Fc region of immunoglobulins might cause a significant cross reaction, even though a "glucagon-similar" sequence is improbable. Preliminary studies in our laboratory indicate that glucagon immunoreactivity can be liberated from the IgG, but conclusive evidence, with biochemical identification of the dissociated material, is still lacking.

Because 10 g (60 μmol) of purified polyclonal IgG per liter interfered in the assay with antisera E7 equivalent to 70–90 ng (20–55 pmol) of glucagon per liter and somewhat less in the assay with antisera 30K, only one molecule of glucagon appears to be bound per ~10^6 molecules of IgG. To investigate whether such binding is to a subset of strongly binding IgG molecules, we assayed the IRG of isoelectrically focused purified polyclonal IgG. The glucagon profile corresponded closely to the IgG concentration profile, with no significant heterogeneity evident in the glucagon profile. Still, some IgG molecules may have a greater binding affinity for glucagon. This was supported by our finding that different monoclonal immunoglobulins of the IgG 1 subclass gave different IRG values. The relative presence of such IgG molecules with various "glucagon-binding tendency" within the total amount of polyclonal IgG cannot be predicted. This would be in agreement with the observed variation of the presence of IRG in our six experiments with the isoelectrically focused solution of high-Mr precipitates of plasma. This finding of variation of the IRG on isoelectric focusing of IgG also may explain the report of Valverde and Villanueva (4), who found no correlation between the concentrations of "big plasma glucagon" and the concentration of polyclonal IgG when they examined patients with high concentrations of "big plasma glucagon."

The IRG with pI between 5 and 6 and close to 5 is still unidentified. By our method, this immunoactivity was apparent in only four of six individuals, and it varied in concentration among these four individuals. We roughly estimate its Mr to be about 200 000.

In conclusion, our study shows that both IgG and some other poorly characterized high-Mr component may substantially interfere in two glucagon assays. Their combined addition to the immunoreactive glucagon values is about 100 ng/L in the glucagon assay with antisera E7 and about 50 ng/L in the assay with antisera 30K. Methods involving extraction of glucagon and precipitation of the interfering high-Mr components before determining the physiological concentration of glucagon have been introduced for antisera 30K and E7 (24, 25). The usefulness and practicality of this extraction have been validated with plasma samples from people subjected to various A-cell stimulation and suppression tests, and the accuracy of the results has been demonstrated in comparison with another carboxyl-terminal-directed antiserum (4305), which does not recognize the high-Mr components (25).

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References