Insulin Radioreceptor Assay for Human Erythrocytes

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Human erythrocytes have specific insulin receptors. A radioreceptor assay for the determination of insulin binding to these receptors is presented. After two passages over a Boyum-type gradient, erythrocytes from freshly collected heparinized blood were isolated and 3.5 × 10⁸ erythrocytes per milliliter were incubated for 2.5 h in a modified pH 8.0 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate buffer, iodinated insulin (80 pg/ml), and a range of unlabeled insulin concentrations (0 to 1 × 10⁵ ng/ml). The incubation was terminated by pipetting 200 μl of the incubated suspension onto 200 μl of buffer and 200 μl of dibutyl phthalate in pre-chilled microcentrifuge tubes. After centrifugation, the supernatant fluid was aspirated, leaving about 0.1 of the dibutyl phthalate on the cell pellets. The percentage of [¹²⁵I]insulin bound was determined after radioactivity of the cell pellets was measured in a gamma counter. Under these conditions 11 normal volunteers demonstrated a mean of 7.2 ± 0.44% insulin bound specifically to 3.5 × 10⁸ cells. The non-specific binding varied from 8–17% of the total insulin bound. Further, a linear increase of specific binding from 1.35 to 13.55% was observed when the cell concentration was increased from 0.72 to 7.2 × 10⁸ cells per milliliter, respectively. Insulin, 100 ng/ml, from several animal species inhibited more than half of the binding of porcine [¹²⁵I]-labeled insulin. Bovine glucagon inhibited 9.8% and bovine somatotropin inhibited 1.1%, whereas desalame-desasparagine insulin and human chorionicadotropin (10 int units) did not inhibit binding of [¹²⁵I]-labeled insulin. For seven duplicates done on a single assay, the CV was 16.1%, whereas that for 11 assays done on different subjects and on different days was 10.7%. Receptor assays utilizing this technique thus have sufficient specificity and sensitivity to be used for further clinical diagnostic and investigative studies of insulin receptors on human erythrocytes.

Additional Keyphrases: normal values - comparative studies - hormones - radio assay - specificity studies

In 1960 Yalow and Berson (1) presented data which firmly established a basis for clinical evaluation of circulating hormones by radioimmunoassay. It was this competitive binding radioimmunoassay which led to the development of radioreceptor assays as a means of directly measuring the cell membrane binding sites for peptide hormones in man.

During the past six years, insulin receptors have been extensively investigated. Initial human insulin radioreceptor assays as reported by Gavin et al. (2) were done on lymphocytes, other mononucleocytes, erythrocytes, and granulocytes. Compared to the mononuclear cells, studies of erythrocytes and granulocytes reported under their initial conditions showed a low percentage of total specific binding, i.e., a low percentage of insulin binding when the nonspecific binding was subtracted from the total insulin binding. Most insulin receptor studies on human circulating cells have thus been done on the monocytes. With binding characteristics similar to the hepatocytes and adipocytes, the monocytes were shown to have insulin receptors that reflected insulin sensitivity or resistance. Using the mononuclear cells, a defect in insulin binding was demonstrated in insulin-resistant obese patients (3). In these patients, a fluctuation in the affinity and concentration of the insulin receptors was shown (4). Further, when the diet of these patients was restricted, the receptors increased to match their new insulin-sensitive states. A decrease in receptors on mononuclear cells has also been demonstrated in diabetics (5, 6).

Monocytes and other mononuclear cells for insulin receptor studies are isolated after leukopheresis. If the patient is to have receptor studied in detail and in duplicate, 150 to 500 ml of whole blood must be removed in a closed sterile system. Then the time-consuming leukopheresis is done, to obtain a buffy coat that finally yields from 70 × 10⁶ to 400 × 10⁶ mononuclear cells. The erythrocytes and plasma are then returned to the patient. Removal of the blood, leukopheresis, and return of the blood are not only time consuming but also require a physician, a trained nurse, or a trained technician. Further, because human blood is being removed and returned, care must be taken to keep individual samples separate until they are infused. This limits the number of patients whose blood can be leukopheresed and isolated in any one day. Thus, under these conditions, assays of insulin receptors on freshly isolated circulating cells are not readily available for clinical purposes.

The present study presents a technique for measuring insulin receptors on the freshly isolated circulating...
human erythrocyte, a cell which can be easily obtained and quickly isolated in large quantities. From 10 ml of whole blood obtained in a single evacuated blood-collection tube, multiple studies can be done on each patient.

Materials and Methods

Materials

Purified porcine insulin (Lot 8GN814) was obtained from Elano Laboratories, a division of Eli Lilly Research Laboratories, Indianapolis, Ind., and was used for iodination as well as unlabeled standard in the binding studies.

Somatotropin (growth hormone), glucagon, and human chorionicsomatotropin were purchased from Sigma Chemical Co. Guinea pig insulin was a gift of Dr. C. C. Yip of Toronto, Canada. Rabbit, bovine, and ovine insulins and bovine proinsulin were generous gifts from Dr. Ronald Chance of Eli Lilly Research Laboratories.

Bovine serum albumin (fraction V) was obtained from Pentex. Carrier free Na$_{125}$I was purchased from Amersham Searle. Dibutyl phthalate (density 1.043) was obtained from Aldrich Chemical Co. Microfuge B, Microfuge tubes, and other accessories were obtained from Beckman Instruments, Inc. Other chemicals were of reagent grade.

Iodination of Insulin

Insulin was iodinated by reacting purified pork insulin with Na$_{125}$I and Chloramine T in equimolar ratios. Five micrograms of insulin in 5 µl of phosphate buffer (0.3 mol/liter, pH 7.4) was added into a Microfuge tube (polystyrene) of 1.5-ml capacity containing 100 µl of the phosphate buffer, 30 µl (130 ng = 1.5 mCi) of Na$_{125}$I, and 5 µl of Chloramine T (50 µg/ml). After about 30 s an aliquot from the reaction mixture was checked for insulin radioactivity. If iodination was less than 50%, an additional 1–2 µl of Chloramine T was added until incorporation of radioactivity exceeded 50% of the total. Then 10 µl of sodium metabisulfite (16 µg/ml) was added to stop the reaction.

The reaction mixture was applied on a preequilibrated Sephadex G-50 (fine) column (9 × 60 cm) with phosphate buffer (pH 7.4, 30 mmol/liter, plus 1 g of bovine serum albumin per liter) in the cold room. The reaction vessel was washed twice with 180 µl of the same buffer and 1-ml fractions were collected. The elution pattern showed three peaks. The fractions corresponding to the iodinated monomer insulin peak were pooled and rechromatographed through the same column; fractions corresponding to the other two peaks were discarded. Then the fractions corresponding to the peak of iodinated monomer insulin were again collected. These pooled fractions were divided and frozen at −15 °C until used. The specific activity of this preparation varied from 130 to 180 Ci/g. This procedure was adopted from Freychet et al. (7), a modified method of Hunter and Greenwood (8). The bioactivity of the iodinated insulin has been extensively discussed (7). For many of the first experiments $^{125}$I-labeled insulin iodinated by the above procedure was kindly supplied by Jesse Roth (NIH).

Buffer G

The composition of this pH 8.0 buffer system was determined after a series of experiments utilizing various constituents chosen from reported insulin receptor-insulin interaction studies. In mmol/liter, it is: tris(hydroxymethyl)methylamine, 50; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 50; MgCl$_2$, 6H$_2$O, 10; CaCl$_2$, 10; ethylenediaminetetraacetic acid, 2; dextrose, 10; NaCl, 50; and KCl, 5 (in addition to albumin, 1 g/liter). This buffer has a calculated osmolarity of 286 mOsm.

Preparation of Erythrocytes for Binding Studies

Fresh human blood, 10 ml, was drawn in a heparinized (green top) Vacutainer tube (containing 143 USP units of sodium heparin per tube; Becton-Dickinson Co.). After centrifugation of the blood (10 min, 400 × g, 20 °C) the plasma was aspirated and the cell pellet was mixed with one part of physiological saline and then layered on 3 ml of Hypaque (33.9%–Ficoll (9%) mixture (1/2.4 by vol) in a glass tube, as described by Boyum (9). After centrifuging at 400 × g for 20 min at 20 °C, the saline monocyte, Hypaque–Ficoll, granulocyte phases, and the upper layer of the erythrocyte phase were aspirated. After suspending the cell pellet with an equal volume of saline, the above procedure was repeated. The erythrocyte pellet was then resuspended in two parts of buffer G, to equilibrate the cells. After the cell suspension was centrifuged (10 min, 400 × g, 4 °C), the buffer was aspirated and the cell pellet resuspended with a volume of buffer G which resulted in a suspension containing 4.4 × 10$^6$ cells per milliliter, more than 95% of which were viable cells as determined by the trypan blue dye-exclusion technique (10). Erythrocyte counts, hematocrit, hemoglobin, and erythrocyte indices (Table 1) were determined with a Coulter counter (Model S Senior), which was standardized three times before counting each specimen.

Preparation of Insulin Standards

Stock solution containing 1 mg of insulin per milliliter of buffer G was prepared, aliquoted, frozen, and stored. From these aliquoted solutions, serial dilutions were made weekly and, when not in use, stored at 4 °C.

Binding Studies

Binding of $^{125}$I-labeled insulin to human erythrocytes was determined by incubating a 400-µl cell suspension (1.75 × 10$^9$ cells in buffer G), 40 pg of $^{125}$I-labeled insulin (in 25 µl of buffer) and various amounts of unlabeled insulin (0 to 0.5 × 10$^5$ ng) in a total volume of 0.5 ml (protocol, Table 2).

After incubating at 15 °C for 2.5 h, 200 µl of the incubated suspension were aliquoted into pre-chilled Microfuge tubes containing 200 µl of buffer G and 200 µl of dibutyl phthalate (Figure 1). These tubes were then
Table 1. Hematological Properties of Erythrocyte Suspensions (from 10 Persons) Used in Receptor Assays

<table>
<thead>
<tr>
<th>Hb g/liter</th>
<th>MCV a</th>
<th>MCH b</th>
<th>MCHC c</th>
<th>Hct d</th>
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<td>29</td>
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<td>39</td>
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<td></td>
<td>132.3</td>
<td>84.45</td>
<td>28.82</td>
<td>34.27</td>
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SD
6.1       5.28  2.40  1.10  1.17

a Mean cell vol, in fl.
b Mean cell hemoglobin, in pg.
c Mean cell hemoglobin concn, in per cent.
d Hematocrit, in per cent.

centrifuged in a Beckman Microfuge B (kept in a 4 °C cold room) for 2.5 min. The buffer and dibutyl phthalate layers were aspirated with a Pasteur pipet, leaving about 0.1 of the dibutyl phthalate on the pellet.

During aspiration, formation of bubbles containing trapped free 125I-labeled insulin in the dibutyl phthalate phase can be avoided by aspirating with a Pasteur pipet that has an uneven tip. It is essential to remove these bubbles to prevent a false-positive binding of 125I-labeled insulin. To determine the bound radioactivity, the tip of the Microfuge tube containing the cell pellet was cut with a heated scalpel and counted in a gamma counter (Searle, Model 1185). The radioactivity bound to the cells was determined by the following:

\[
\text{Percentage of radioactivity bound} = \left( \frac{\text{erythrocyte pellet radioactivity}}{\text{total radioactivity (in 200 μl of the incubated cell suspension)}} \right) \times 100
\]

The percentage of specific insulin bound (3) at each concentration of unlabeled insulin was determined by subtracting the percentage of 125I-labeled insulin bound at 1 × 10^{-5} ng/ml of unlabeled insulin from the total percentage of 125I-labeled insulin bound at each concentration of unlabeled insulin.

Table 2. Protocol for the Radioreceptor Assay a

<table>
<thead>
<tr>
<th>Tube No. (Blank)</th>
<th>Buffer G, μl</th>
<th>Conc of stock insulin standard, ng/μl</th>
<th>Vol of insulin standard to be added, μl</th>
<th>Final insulin concn, ng/ml</th>
<th>125I-labeled insulin, ml</th>
<th>Cell suspension, ml</th>
<th>Final vol, μl</th>
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<tr>
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<td>500</td>
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<tr>
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<td>500</td>
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<td>50</td>
<td>100 000</td>
<td>25</td>
<td>400</td>
<td>500</td>
</tr>
</tbody>
</table>

a Reagents were added in the order indicated in the protocol, starting with "Buffer G" and ending with "Cell suspension."

b Unlabeled insulin.
Results and Discussion

With this method, $3.5 \times 10^8$ human erythrocytes from each of 11 normal volunteers bound a maximum of 7.2% of the $^{125}$I-labeled insulin (SD 0.44%) (Figure 2). At an insulin concentration of $10^5$ ng/ml, these cells bound only 8–17% of the total $^{125}$I-labeled insulin available. Since circulating human erythrocytes measured with the existing techniques had 42% of the $^{125}$I-labeled insulin bound at 0.5 $\times 10^5$ ng/ml (2), this method provides a means of measuring more specific insulin binding over a wide range of insulin concentrations. With turkey erythrocytes, the binding at 0.5 $\times 10^5$ ng of insulin per milliliter was only 10–20% (11); however, turkey erythrocytes were easily measured by the old technique because they are nucleated and, thus, more dense than human erythrocytes. Separation of the erythrocytes from free insulin at the end of incubation by use of dibutyl phthalate and pre-chilling the Microfuge tubes before layering the incubation suspension is essential to achieve low binding of $^{125}$I-labeled insulin at the highest insulin concentrations.

To demonstrate that the binding of $^{125}$I-labeled insulin was a function of cell concentration—i.e., receptor sites per cell—insulin binding was studied over a 10-fold cell concentration range. When $0.72 \times 10^8$ to $7.2 \times 10^8$ cells were incubated with $^{125}$I-labeled insulin, 1.35 to 13.55%, respectively, the $^{125}$I-labeled insulin was specifically bound (Figure 3). These erythrocytes have receptor sites that are specific for porcine insulin. When the cells were incubated with 100 ng of ovine, rabbit, bovine, or guinea pig insulins, bovine proinsulin, glucagon, desalanne-desasparagine insulin, somatotropin and (or) 10 int. units of choriogonadotropin, less inhibition of $^{125}$I-labeled insulin binding was evident as compared to inhibition of binding by 100 ng of porcine insulin. All the insulins except desalanne-desasparagine insulin demonstrated greater than 50% inhibition. However, glucagon had only 11% and somatotropin 1.3%, whereas desalanne-desasparagine insulin and choroiogonadotropin demonstrated no inhibition of $^{125}$I-labeled porcine insulin (Figure 4).

To establish the precision of this radioreceptor assay in erythrocytes, we have determined the coefficient of variation (CV) on the basis of the amount of unlabeled
insulin required to inhibit 50% of the $^{125}$I-labeled insulin binding to the receptors. The CV was calculated for duplicate values in a single daily assay and from duplicates in assays done on different days (Table 3). The CV for a single assay (seven duplicates) was 16.1% and from assay to assay (11 days) it was 10.7%. Several factors may influence these values. Because calculation of the CV is based on the number of volunteers, the CV varies directly with the number of volunteers. Future studies are planned to define the nature of the influence that the number of volunteers has on the assay-to-assay CV. A further consideration is age variation of cell population, both among volunteers and within a single volunteer. Although the purified erythrocyte suspension contained no reticulocytes, this suspension did contain normal age variants of circulating human erythrocytes.

The technique used for human circulating and cultured monocytes (2, 12, 13), cultured human fibroblasts (2, 14), rat thymocytes (15, 16), hepatocytes (17, 18), myocytes (19), and turkey erythrocytes (11) was inadequate for determining the degree of specific insulin binding. To study the insulin binding by human erythrocytes, it was necessary first to obtain an erythrocyte specimen with few or no reticulocytes, monocytes, granulocytes, or lymphocytes. By two passes through a Boyum gradient (9) and aspiration of all phases except the lower erythrocyte phase, we obtained a cell suspension with only a few lymphocytes, no reticulocytes, no granulocytes, and no monocytes.

Secondly, a physiological buffer that promoted stability of the flexible erythrocyte membrane (20) and its receptors while maintaining the isotonicity was necessary. Preliminary experiments indicated that erythrocyte membrane receptors were best demonstrated in buffer consisting of both Tris and Heps in an equimolar ratio with the addition of Ca$^{2+}$, K$^+$, Na$^+$, and Mg$^{2+}$ to maintain isotonicity.

Finally, the erythrocyte with its bound insulin must be separated from the free insulin at the end of the incubation. Dibutyl phthalate was found to be ideal for this separation because:

(a.) dibutyl phthalate does not affect erythrocyte membrane;
(b.) dibutyl phthalate has a density (1.043) between that of aqueous buffer containing the free insulins (labeled as well as unlabeled) and the erythrocytes, thus distinctly separating the cell pellet from the buffer (Figure 1);
(c.) dibutyl phthalate prevents accidental aspiration of erythrocytes during aspiration of the buffer since human erythrocytes do not pack as tightly as monocytes, thymocytes, hepatocytes, myocytes, or avian erythrocytes;
(d.) dibutyl phthalate does not mix with aqueous buffer and thus minimizes nonspecific binding; and
(e.) dibutyl phthalate left over the cell pellet during aspiration does not affect the actual bound radioactivity.

We have thus presented an assay with which one can do investigational and routine clinical assays for insulin receptors on easily obtained human erythrocytes. This assay system may be applied to the determination of other peptide hormone receptors on human erythrocytes.

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