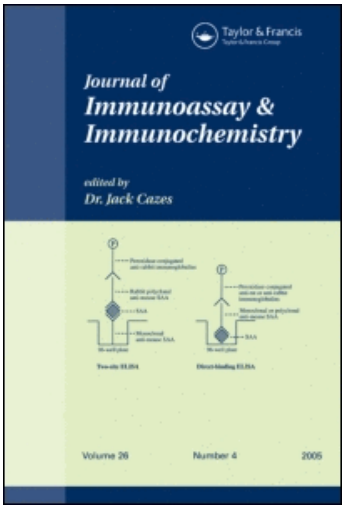

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Radioimmunoassay for Insulin-Like Growth Factor (IGF). II: Interference by Pure IGF-Binding Proteins

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RADIOIMMUNOASSAY FOR INSULIN-LIKE GROWTH FACTOR (IGF) II: INTERFERENCE BY PURE IGF-BINDING PROTEINS

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ABSTRACT

A new radioimmunoassay for insulin-like growth factor-II (IGF-II) is described. Compared to recombinant DNA-derived IGF-II standard, the cross-reactivity of natural or recombinant IGF-I was <1%. The ED_{50} for IGF-II standard was 1.0 ng/ml, and the mean IGF-II level in acid-ethanol-extracted serum from healthy adults was 525±87 ng/ml (SD, n=30). Addition of the IGF binding protein IGFBP-1 (BP-28, PP12) caused dose-dependent inhibition of IGF-II tracer binding to antiserum, increasing to >90% inhibition at 400 ng/ml IGFBP-1. In contrast, the IGF binding protein IGFBP-3 (BP-53) caused approximately 30% inhibition of tracer binding at 20 ng/ml IGFBP-3, with no further inhibition up to 400 ng/ml IGFBP-3. The influence of added IGF binding proteins on IGF-II displacement curves varied depending on both the type and concentration of binding protein added. It is concluded that interference in IGF radioimmunoassays by IGF binding proteins depends both on the types of binding proteins present, and on the IGF concentration, in the test samples.

(KEY WORDS: insulin-like growth factor-II, radioimmunoassay, IGF-binding protein, IGFBP-1, IGFBP-3)

INTRODUCTION

Insulin-like growth factor-II (IGF-II), a single-chain polypeptide of 7.4 kDa with anabolic and mitogenic activities, is

involved in the growth of both normal and neoplastic cells (1-3). The role of this peptide in the induction of hypoglycemia in patients with non-pancreatic tumors has attracted interest recently with the confirmation that both circulating IGF-II, and tumor IGF-II mRNA, are elevated in these subjects (4,5).

Due to the limited availability of pure IGF-II in the past, few laboratories have established IGF-II assays. Radioreceptor assays using microsomal or plasma membranes from rat liver (6,7), rat placenta (8), or sheep placenta (9), as a source of IGF-II receptors, have all shown sufficient specificity and sensitivity to be useful in clinical and experimental studies, but involve the inconvenience of preparing membranes. Several previously described IGF-II antibodies, when used in RIAs, have shown high cross-reactivity (i.e. at least 10%) with the related peptide IGF-I (10-12), or low sensitivity towards IGF-II (13). This paper describes the production of a highly specific, high-affinity antiserum against human IGF-II, and the development of a sensitive RIA using this antiserum.

Both IGF-I and IGF-II circulate in association with specific IGF-binding proteins (14). The nomenclature of these proteins has recently been standardized (15). Although Blum et al. (16) demonstrated that binding proteins present in serum samples could cause interference in an IGF-II RIA, the extent of this interference has never been assessed using purified proteins. Therefore in this study the effect of two well characterized IGF-binding proteins (14), the "amniotic fluid binding protein" IGFBP-1 (also known as BP-28 or placental protein 12) and the growth hormone-dependent binding

protein IGFBP-3 (also known as BP-53), on the RIA for IGF-II, has been investigated.

MATERIALS AND METHODS

Peptides.

Natural IGF-I (Batch I-6) and IGF-II (Batch II-5) were purified from Cohn fraction IV of human plasma as previously described (9). Recombinant DNA-derived IGF-I (recIGF-I) was generously provided by Dr. Dan Burleigh, IMC, Northbrook, IL, and recIGF-II by Eli Lilly and Company, Indianapolis, IN. The human IGF-binding protein IGFBP-1 (BP-28) was purified from pooled amniotic fluid (17), and IGFBP-3 (BP-53) was purified from Cohn fraction IV of human plasma (18).

IGF-II Antiserum.

Natural human IGF-II (200 μg) was coupled to 75 μg ovalbumin (Sigma, Grade III) by adding 100 μl of 25% aqueous glutaraldehyde, diluted 1:10, to the proteins dissolved in 400 μl of 0.22 M sodium phosphate buffer, pH 7.3. After 24 h the reaction mixture was diluted to 1 ml with 0.15 M NaCl and dialyzed against 1 liter of 0.1M NaCl. Two New Zealand white rabbits were injected s.c. at several dorsal sites with IGF-II/ovalbumin containing 100 μg IGF-II, emulsified in Freund's complete adjuvant. A similar dose in incomplete adjuvant was given after 2 weeks. Intramuscular boosts in saline, without adjuvant, were given at 4 and 7 weeks. One week after the final boost animals were bled, one yielding an antiserum

(S2) suitable for use in RIA. To avoid possible effects of IGF-binding proteins in the rabbit antiserum, antibodies were purified on a column of Protein-A Sepharose (Pharmacia, Sydney, NSW) according to the manufacturer's instructions. The recovery of IGF-binding immunoglobulins was quantitative.

RIA for IGF-II.

After testing various buffers at pH values between 6.0 and 9.0, a buffer containing 0.1 M Tris-HCl (pH 8.0), 0.02% sodium azide, 0.25% bovine albumin, was selected. Incubations, in a final volume of 0.5 ml, contained affinity-purified antiserum S2 (1:5000 final dilution), standards or other test substances, and approximately 10,000 cpm of ^{125}I-labeled natural IGF-II, prepared as previously described (9) to a specific activity of 200-300 Ci/g. The standard IGF-II preparation, unless otherwise noted, was recIGF-II, used over the range 0.1 to 10 ng/tube. Incubations were for 16 h at 2 °C, since tracer binding was found to be considerably greater at 2 °C than at 22 °C. Bound tracer was separated from free by centrifugation, after adding 0.5 µl normal rabbit serum and 2 µl goat anti-rabbit IgG (Bioclone, Sydney, NSW), incubating 30 min, then adding 4% (final concentration) polyethylene glycol solution in 0.15 M NaCl.

Serum samples from 30 healthy adult volunteers (18 males), aged 21-66 years, were obtained from the Red Cross Blood Bank, Sydney. To remove binding proteins, each sample was extracted before assay with 4 volumes of acidified ethanol, according to the method of Daughaday et al. (19). The neutralized extracts (diluted 1:7

compared to the original serum) were diluted a further 5-fold in RIA buffer, and 50 µl of each diluted extract was assayed in duplicate.

RESULTS

Fig. 1 shows displacement curves for the IGF-II RIA, using recombinant and natural IGF-I and IGF-II preparations. The mean Bo binding was 47.1±3.6% of total radioactivity (±SD, n=4), with nonspecific binding (measured in the absence of first antibody) of 5.9±0.7%. For recIGF-II, the preparation routinely used as a standard, the ED_{50} was 0.52±0.05 ng/tube (i.e. 1.04±0.10 ng/ml), and the lowest standard, 0.1 ng/tube, gave a B/Bo value of 0.95±0.02. As determined by Scatchard analysis, the affinity constant for IGF-II binding to antibody was $2.3\pm0.4 \times 10^{10}$ M^{-1}. Natural human IGF-II prepared in this laboratory had 70% of the activity of recIGF-II, whereas natural IGF-I showed only 0.7% crossreactivity, and recIGF-I 0.2% crossreactivity, compared to the recIGF-II standard. Thirty serum samples from healthy human volunteers had an apparent mean IGF-II level (±SD) of 525±87 ng/ml, giving a reference range (mean±2SD) of 350-700 ng/ml.

When increasing concentrations of pure IGF binding proteins, IGFBP-1 or IGFBP-3, were present during the incubations of IGF-II with antiserum, the pattern of inhibition of tracer binding to the antiserum was distinctly different for the two binding proteins (Fig. 2). IGFBP-1 gave a dose-dependent inhibition curve up to a concentration of at least 200 ng/tube (400 ng/ml), at which point more than 90% of tracer binding to antibody was inhibited. In contrast,

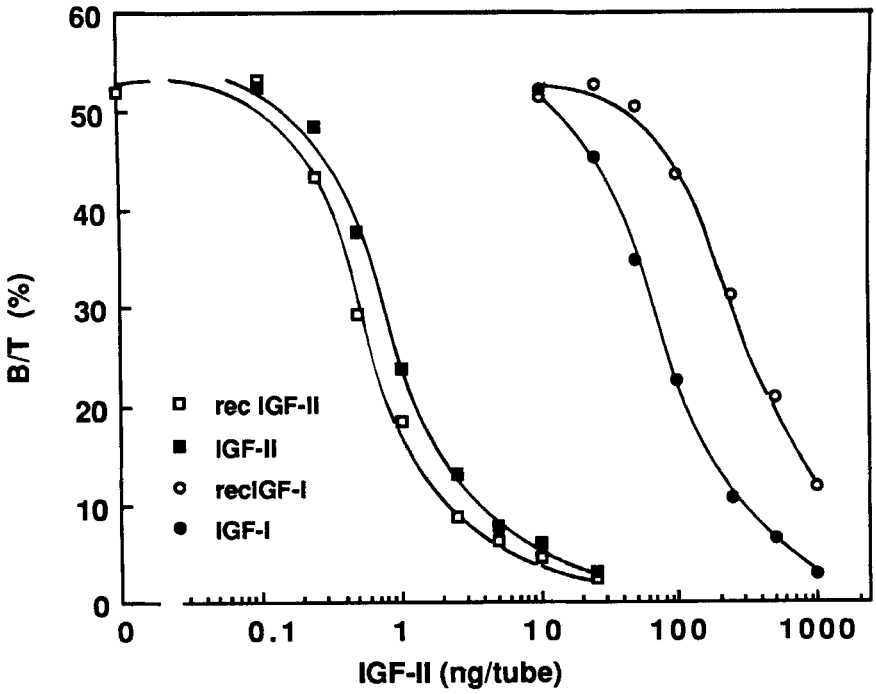

FIGURE 1. Displacement of IGF-II tracer from antiserum S2 by recombinant DNA-derived (rec) IGF-II, natural IGF-II, rec IGF-I, and natural IGF-I. The reaction volume was 0.5 ml. B/T represents antibody-bound radioactivity expressed as a percentage of total radioactivity,

increasing concentrations of IGFBP-3 caused dose-dependent inhibition only up to a concentration of 10 ng/tube (20 ng/ml), where approximately one third of specific tracer binding was inhibited. In repeated experiments, the addition of IGFBP-3 up to 200 ng/tube (400 ng/ml) gave no further inhibition of binding.

To test the effect of the binding proteins over a wide range of IGF-II concentrations, IGF-II displacement curves were repeated

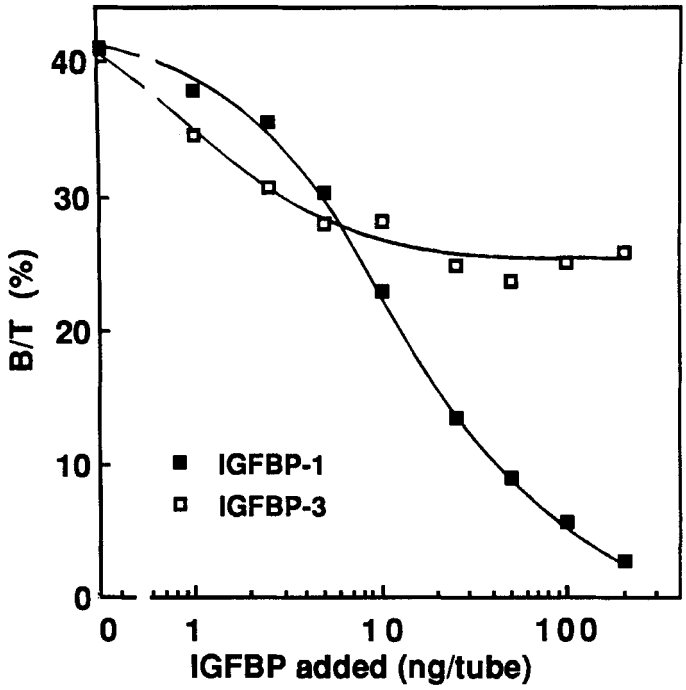

FIGURE 2. Inhibition of specific IGF-II tracer binding to antiserum S2 by increasing concentrations of pure IGF binding proteins, IGFBP-1 and IGFBP-3.

in the presence of various concentrations of IGFBP-1 or IGFBP-3. As seen in Fig. 2, increasing concentrations of IGFBP-1 increasingly inhibited IGF-II tracer binding in the absence of added unlabeled IGF-II (Fig. 3). As the total IGF-II concentration was increased by adding unlabeled peptide, a paradoxical increase in IGF-II binding to the antibody was observed. At higher IGF-II concentrations, the curves became superimposable with the displacement curve seen in the absence of binding proteins. Because of the extreme inhibition of

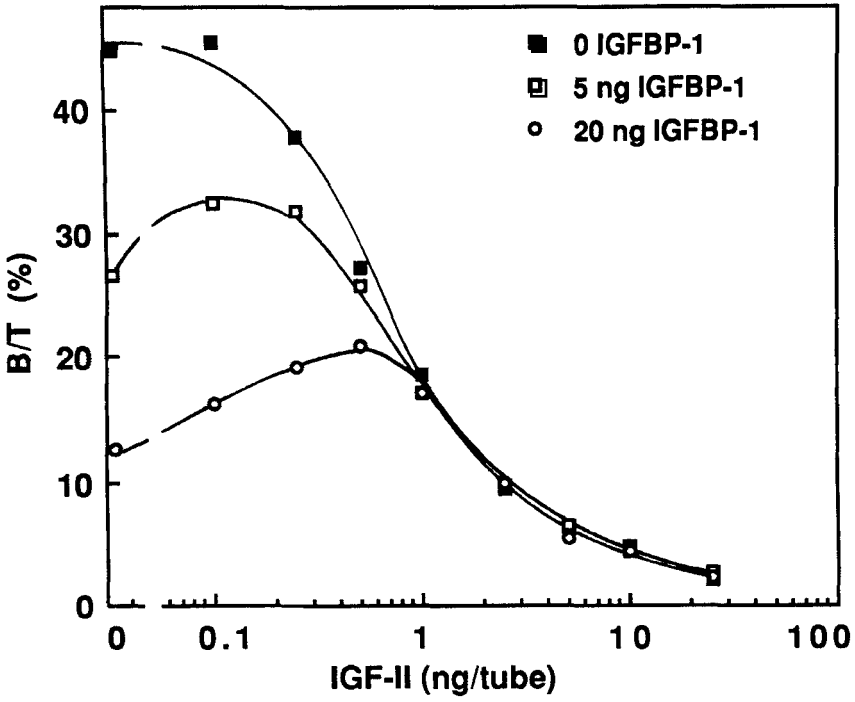

FIGURE 3. Effect of increasing concentrations of pure IGFBP-1 on the IGF-II displacement curve.

binding seen in the presence of high IGFBP-1 concentrations, only concentrations up to 20 ng/tube were tested.

The effect of IGFBP-3 on IGF-II displacement curves was quite different. As seen in Fig. 2, specific IGF-II tracer binding in the absence of unlabeled IGF-II was inhibited to a similar extent (approximately 30%) by 5, 20, or 100 ng/ml IGFBP-3 (Fig. 4). The addition of increasing IGFBP-3 to IGF-II displacement curves gave an unexpected series of curves. At 5 ng/tube, the sensitivity to IGF-II appeared slightly decreased (i.e. the displacement curve was shifted

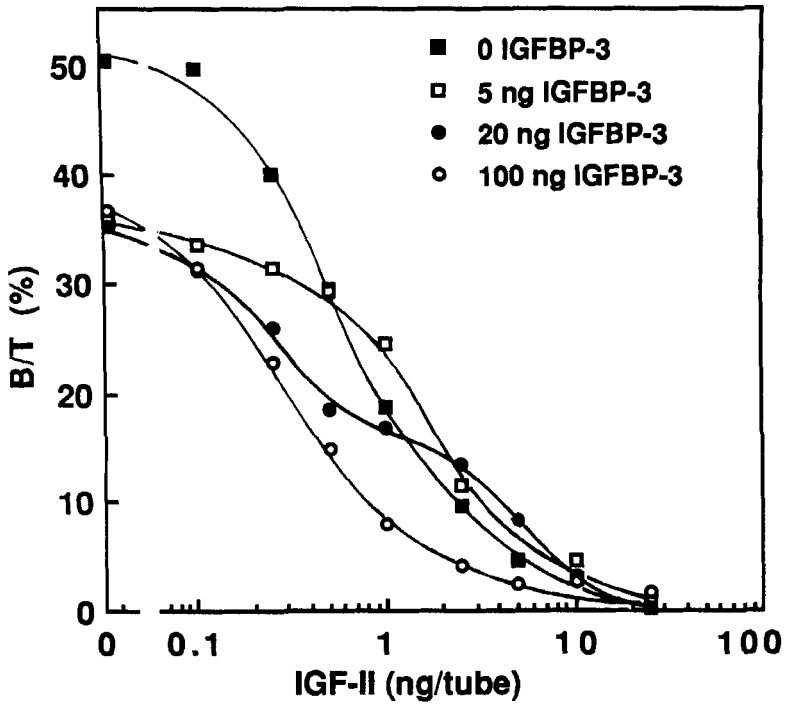

FIGURE 4. Effect of increasing concentrations of pure IGFBP-3 on the IGF-II displacement curve.

to the right). At 100 ng/tube, the apparent sensitivity was slightly increased (i.e. the displacement curve was shifted to the left). An intermediate, biphasic inhibition curve was seen in the presence of 20 ng/tube IGFBP-3 (Fig. 4). Similar results were seen in three separate experiments.

DISCUSSION

This paper describes a new IGF-II RIA which compares favorably with most of the previously described radioligand assays

for IGF-II. The apparent mean serum IGF-II level determined in 30 normal adults (525 ng/ml) is comparable to, or slightly lower than, the range of mean values reported in other studies (2,9,16). Furthermore, the normal range (mean±2SD) obtained with this assay, 350-700 ng/ml, falls within the range of 250-900 ng/ml previously obtained in this laboratory for 100 samples using an ovine placental membrane radioreceptor assay (9).

The potential for interference by IGF binding proteins in IGF RIAs was first demonstrated by Furlanetto et al. (20) for the IGF-I RIA, and has since been discussed in detail (2). For the IGF-II RIA, Blum et al. (16) showed that residual binding proteins present in acid-ethanol extracts of some serum samples (e.g. umbilical cord sera, but not normal adult sera) could give falsely elevated IGF-II values. Furthermore, they elegantly demonstrated that the addition of IGF-I, to occupy IGF-binding sites on the binding proteins, effectively removed the interference in cord serum samples if a highly specific antiserum was used (16).

As recently reviewed (14), the IGF-binding proteins characterized to date fall into three distinct groups (15), based on primary structure and binding specificity. Proteins of two of these groups, IGFBP-1 and IGFBP-3, have been identified by specific RIAs in the human circulation (21,22). The growth hormone-dependent protein, IGFBP-3, is effectively removed from human serum by acid-ethanol extraction (22); however, it is not known to what extent this method removes IGFBP-1. Since IGFBP-1 levels are high in cord serum (21), it is likely that the protein in acid-ethanol extracts that was reported to interfere in the IGF-II RIA (16) was IGFBP-1.

In the present study, pure IGFBP-1 and IGFBP-3 were tested for their interference in the IGF-II RIA. Interestingly, the pattern of interference for the two proteins was quite different. Although the binding affinity of IGFBP-3 for IGF-II, 3 x 10^{10} M^{-1} (18), is very similar to the affinity of antiserum S2, and an order of magnitude higher than the affinity of IGFBP-1 for this peptide (17), IGFBP-1 inhibited IGF-II tracer binding to the IGF-II antiserum to a much greater extent than IGFBP-3. The precise reason for this discrepancy is unclear, but presumably relates to the relative locations of the protein-binding and antibody-binding determinants on IGF-II. When tested over a range of IGF-II and binding protein concentrations, the nature and extent of interference by IGFBP-1 and IGFBP-3 showed even greater differences. Thus, in the presence of IGFBP-1, IGF-II tracer binding to antiserum was actually increased by the addition of unlabeled IGF-II, which presumably displaced the tracer from the binding protein, making it more accessible to the antibody.

This study demonstrates that the interference in the IGF-II RIA by IGF binding proteins is unpredictable, being a function of the type and concentration of the binding proteins present, as well as the concentration of IGF-II in the incubation mixture. It is likely that IGF-I RIAs would be affected similarly. Since the serum concentrations of various binding proteins are regulated in a variety of ways, e.g. age, growth hormone status, metabolic state etc. (14), this study emphasizes that removal of binding protein from serum samples prior to assay is important to ensure meaningful results.

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