Radioimmunological Determination of Cellular Retinol-binding Protein in Human Tissue Extracts

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ABSTRACT

A sensitive and specific radioimmunoassay was developed for human cellular retinol-binding protein (CRBP) using antibodies raised in rabbits against purified human liver CRBP. The assay could determine concentrations of CRBP in extracts down to 8 µg/liter. CRBP could be demonstrated in all tissues investigated except in serum, and the same immunoreactive CRBP seemed to be present in all tissues. The highest concentrations were found in the gonads. In the gastrointestinal tract, the small intestine had a higher concentration than did stomach or colon. Muscle and skin had low concentrations of CRBP.

INTRODUCTION

A CRBP,2 which is distinct from the plasma retinol-binding protein, has recently been isolated from animal (9, 14, 19, 21–23) and human (6, 12) tissues and partially characterized. From the limited sequence data available, CRBP seems to have a highly conserved structure (5, 6, 20). It is probably not tissue specific; i.e., the same molecular form of CRBP seems to occur in all tissues within each species (15). CRBP occurs in many experimental tumors (4) and malignant human tumors (4), and in some human tumors, the CRBP concentration is either markedly higher or lower (17, 18) than in surrounding normal tissue. The role of CRBP is presently unknown, but it seems to mediate the biological effects of its ligand as possible prophylactic and/or therapeutic agents in cancer (24), there is also a great interest for determination of CRBP in tumors as a possible means to judge the sensitivity of the tumors to treatment with retinoids.

Until now, CRBP has been determined by binding assay (13) which is complicated and at best semiquantitative. For rat tissues, the problem was recently alleviated by the development of specific and sensitive radioimmunoassays (1, 16). In this paper, we describe a radioimmunoassay for human CRBP and give the levels for CRBP in various human tissues.

MATERIALS AND METHODS

Purification of Human CRBP. Human CRBP was purified from human liver as described previously (6). The CRBP which was to be labeled with 125I (Na125I, carrier free, 17 Ci/mg; New England Nuclear, Dreieich, West Germany) according to the method of Bolton and Hunter (2). The labeled CRBP was separated from other reaction products by Sephadex G-75 chromatography (column dimensions, 0.9 x 60 cm) in 0.1 M borate buffer, pH 8.5, containing gelatin (1 g/liter; Favoritgelatin; Töröf & Co., Ekerö, Sweden). The fractions were collected in plastic tubes to which had been added in advance β-mercaptoethanol (0.05 mM), EDTA (0.05 mM), and aprotinin (5 mg/liter; Sigma Chemical Co., St. Louis, MO). After separation, bovine serum albumin (final concentration, 5 g/liter) was added to the fractions. The material eluting at Kav 0.33 (corresponding to the Kav of unlabeled CRBP) was used for radioimmunoassay. The specific radioactivity was around 10 µCi/µg CRBP. About 80% of this labeled CRBP was precipitable with excess specific antibody. The tracer was stored at 4°C and could be used for radioimmunoassay for between 1 and 2 months.

Immunization. Rabbits were immunized by multisite intradermal injections on the back with 0.5 mg CRBP in complete Freund's adjuvant every 14 days for 2 months and thereafter once every month. After the first month, the rabbits were bled 7 to 10 days after each booster. The presence of antibodies to CRBP was determined by testing the ability of antiserum dilutions to precipitate labeled CRBP. The first antibodies were detected 1 month after the start of immunization.

The antisera were fractionated by DEAE-Sepharose chromatography, and the immunoglobulin fractions were tested for antibodies to CRBP. The antibody dilution that precipitated about one-half of the tracer was chosen for the radioimmunoassay.

Radioimmunoassay. The assay system (final volume, 500 µl) consisted of 50 µl labeled CRBP (about 15,000 cpm), 100 µl antibody dilution, and 250 µl sample or standard. To the standards were also added 100 µl normal rabbit serum diluted 1/400. All dilutions were done with 0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, gelatin (1 g/liter), Triton X-100 (2 g/liter), and 5 mM EDTA.

After incubation at 4°C for 48 hr, 500 µl goat anti-rabbit IgG antiserum diluted 1/25 in 0.01 M sodium phosphate, pH 7.4, containing 0.14 M NaCl, 5 mM EDTA, Triton X-100 (2 g/liter), and Polyethylene Glycol 6000 (50 g/liter) (w/v) were added. After mixing, the samples were incubated at 4°C for an additional 24 hr and then centrifuged at 4°C for 15 min at 2000 x g in a MSE Coolspin centrifuge. The supernatants were decanted, and the radioactivity of the precipitates was measured in a 16-channel NE 1600 gamma counter (Nuclear Enterprises, Sighthill, Scotland) connected to a computerized evaluation program.

Preparation of Tissue Extract. Small tissue samples were obtained at autopsy from 7 men aged 45 to 60 years and 8 women aged 69 to 85 years. Three cases had died from cancer, and the others died from cardiovascular causes. The specimens were kept at -20°C in closed vials for periods varying from a few days to four months. Just prior to homogenization, the tissue specimens were cut while frozen in slices as thin as possible, and care was taken to obtain as representative samples as possible of the tissue in question. The sliced tissue was then homogenized (on ice) in 2 ml 0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, Triton X-100 (2 g/liter), 5 mM EDTA, and aprotinin (140 mg/liter) (Sigma Chemical Co., St. Louis, MO) per g tissue using a Polytron homogenizer (Kinematica GMB, Luzern, Switzerland). The homogenates were centrifuged for 5 to 10 min at 500 x g, and the supernatants were used for assay of CRBP after appropriate dilution with the above buffer containing gelatin (1 g/liter).

Electrophoretic Methods. Polyacrylamide slab gel electrophoresis...
was performed as described previously (11). After staining with Coomassie brilliant blue, the gels were washed in 1% (v/v) glycerol/10% (v/v) acetic acid in water and then dried on cellophane using a slab gel drier (Model 2003; LKB Produkter, Bromma, Sweden). The dried gels were in some cases subjected to autoradiography.

Other Methods. Protein determinations of the CRBP solutions used as calibration standards were performed using the method of Lowry et al. (10), and the protein concentration in the tissue extracts was determined according to the method of Bradford (3). In both cases, bovine serum albumin was used as calibration standard.

RESULTS

The tracer had an electrophoretic mobility similar to that of unlabeled CRBP (Fig. 1). Also, the radioactive material precipitated by excess antibody showed one main band corresponding to the mobility of the tracer CRBP and unlabeled CRBP (Fig. 1). It can also be seen that the tracer was virtually free from contaminants. The standard curve had its highest sensitivity below the concentration of 100 µg/l (Chart 1). The 125I-CRBP

![Chart 1. Radioimmunoassay standard curve for human liver CRBP. The concentration on the X axis refers to the CRBP concentration in the respective standard solutions. The Y axis gives the percentage of bound 125I radioactivity in the standards expressed as a percentage of the zero standard. With the antisera dilution used, usually 50 to 65% of the radioactivity was bound in the zero standard.](chart1.png)

The displacement curves obtained with the tissue extracts were similar to that of purified CRBP (Chart 2). The coefficient of variation calculated for the controls included in every assay was 14% at the level of 20 µg CRBP/liter extract.

The CRBP concentrations of various human tissue extracts are shown in Table 1. The samples from the intestine were from jejunum and colon sigmoideum. They were cut obliquely, and the values thus represent an average of the CRBP content of the various layers. Human testis and ovary had the highest CRBP concentrations, followed by the liver and the small intestine. Other tissues, like muscle and skin, had low levels. Serum contained very little or no CRBP. For comparison, data for rat tissues (1, 16) are also given in Table 1.

DISCUSSION

Like others (1, 16), we had great problems in raising a good antiserum to CRBP in rabbits. Several ways to augment the antibody response, i.e., polymerization with glutaraldehyde or coupling to bovine or rabbit serum albumin, were tried without any apparent success. The final antiserum obtained had low titer
obtained using Sephadex G-75 for purification of the labeled tracer was precipitatable to >80% with excess antibody, and the parent molecular weight of about 10,000, which was not visible as determined by slab gel electrophoresis and subsequent autoradiography. As expected, the amount of CRBP radioactivity corresponding to the molecular weight of unlabeled CRBP (Fig. 1). The calculated specific radioactivity, about 10 μCi/g, was similar to that reported by others (1).

To test the possibility of immunological cross-reaction between CRBP and the M, 10,000 impurity, a preparation containing both labeled CRBP and labeled M, 10,000 impurity was used as tracer in a radioimmunoassay standard curve (not shown). CRBP purified by hydroxyapatite chromatography (to remove the M, 10,000 impurity) was used as standard. The resulting antibody precipitate was then subjected to slab gel electrophoresis and autoradiography. As expected, the amount of CRBP radioactivity in the precipitate decreased with increasing addition of unlabeled CRBP. The amount of precipitated M, 10,000 radioactivity was, however, not influenced, which indicates no cross-reactivity between CRBP and M, 10,000 impurity. That the dilution curves obtained with the tissue extracts (Chart 2) were parallel to the standard curve demonstrates the absence of components showing immunochemical cross-reactivity with CRBP in the tissue tested.

The concentrations of CRBP in human tissues are shown in Table 1. Due to the nature of the material, the results should be considered minimum values. The individual variation in our material was relatively great. However, this also seems to be the case in rats (1, 15).

In humans, the CRBP levels were highest in the gonads, the liver, and the small intestine. Compared to the rat tissue data of Ong et al. (16), the present values were generally higher with the exception of lung and kidney, where the values were relatively similar. In comparison with the data of Adachi et al. (1), our values were relatively similar for most tissues, including liver, but higher for the gonads and lower for kidney (Table 1).

Adachi et al. (1) found relatively high CRBP concentration in serum. We tested 10 different sera diluted and undiluted but were unable to detect any CRBP in accord with the findings of Ong et al. (16). The reason for the discrepant results with serum is unknown at present.

If one considers the possibility of species differences, differences with respect to age and sex, and differences due to the difficulty in getting representative samples and to autolytic processes in the human tissue specimens, the similarities between the results of this study and the results of Adachi et al. (1), which were obtained with methods similar to our, are relatively good. As the displacement curves obtained with tissue extracts were parallel to the standard curve, we assume that the same antigen was present in all assayed tissues as seems to be the case in the rat (15).

The lower absolute figures obtained by Ong et al. (16) (Table 1) may be due partly to their extraction procedure. The final extract which they use for the assay is a 630,000 × g minimum supernatant, while Adachi et al. (1) and we use low centrifugal force or no centrifugation at all. It is well known that, during homogenization, vesicles form which may trap soluble protein. Consequently, low or no centrifugal force in combination with detergents (which breaks up vesicles and aggregates (1)) should be preferred if high recovery is desired.

The function of CRBP is unknown at present, but it is certainly involved in retinol action at the cellular level, as the effect of retinol analogues in tissue culture is related to their ability to bind to CRBP (4).

However, regardless of the exact function of CRBP, the varying concentrations of CRBP in different tissues might be regarded as an index of the relative retinol dependency of the various tissues or indicate the amount of retinol handled in the respective tissues. Thus, in humans, gonads, liver, small intestine, stomach, and kidney seem to need and/or handle high amounts of retinol, while muscle and skin need and/or handle less (Table 1). In the gastrointestinal tract, the small intestine seems to need and/or handle more retinol than the stomach and the colon, possibly reflecting the fact that retinol absorption and esterification occur in the former (8).

Human cancers have been reported to contain either markedly higher (17, 18) or markedly lower (18) concentrations of CRBP compared to normal tissue, suggesting these tumors are more or less retinol dependent and therefore susceptible or not susceptible to treatment with retinol analogues. For the future use of retinoids in cancer treatment, it will probably be necessary to know the concentration of CRBP in the tumor in order to judge if retinoid therapy is likely to be successful, in much the same way as determinations of estrogen receptor in mammary cancer are used today.

REFERENCES
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