Monoclonal antibody studies of the antigenic determinants of human plasma retinol-binding protein

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A battery of monoclonal antibodies (MoAbs) against human retinol-binding protein (RBP) was produced to obtain useful probes for the study of the antigenic determinants of RBP. The 12 antibodies all reacted with human RBP by immunoblotting. Based on antibody cross-competition radioimmunoassays, four distinct and different groups of antibodies were identified: group I, 1A4 and 2F4; group II, 1G10, 5C5, 6F4, and 7G3; group III, 5H6, 6C7, 10G5, and 14E3; and group IV, 5H9 and 13A1. Information about the epitopes of RBP recognized by these MoAbs was obtained by testing the reactivity of each antibody with human, rabbit, and rat RBPs by immunoblotting. Group I and group IV antibodies reacted to a similar extent with human, rabbit, and rat RBPs. Group II antibodies reacted strongly with human and rabbit RBPs, but reacted very weakly with rat RBP. Group III antibodies reacted strongly with human RBP, but did not react with rabbit or rat RBP. Thus, the epitopes for group I and group IV antibodies appear to be regions of the RBP molecule that are conserved across the three species, whereas group III antibodies recognized only human RBP. In a preliminary study, the reactivity of each antibody with purified cyanogen bromide fragments of RBP was tested by slot immunoblotting. None of the MoAbs reacted with any of the cyanogen bromide fragments. This study shows that MoAbs specific for at least four different regions of the RBP molecule can be produced; hence, RBP contains at least four major antigenic domains.

Keywords: Retinol-binding protein; monoclonal antibodies; antigenic determinants; vitamin A; retinoids

Introduction

Retinol (vitamin A) circulates in plasma bound to a specific transport protein, retinol-binding protein (RBP). This protein has been characterized extensively since its original isolation from human plasma,[1] and a great deal is known about its structure, physiologic functions, and metabolic regulation (see ref. 2 for review and references). Retinol-binding protein is a single polypeptide chain of 182 amino acids of known sequence[3] with a molecular weight of approximately 21,000.[1-3] The protein has a single binding site for one molecule of retinol and circulates mainly as the retinol-RBP complex, or holo-RBP.[1,2] Holo-RBP interacts in plasma with another protein, transthyretin (TTR), and normally circulates as a 1:1 molar RBP-TTR complex.[1,2] The three-dimensional structure of RBP has been determined by x-ray crystallography;[4] RBP was found to have an eight-stranded beta-barrel core that completely encapsulates the retinol molecule.

Retinol-binding protein is involved in several molecular interactions of physiologic importance. First, the interaction of RBP with the ligand retinol is central to its physiologic role. Thus, RBP serves to transport retinol in plasma and to deliver it to tissues throughout the body. In particular, RBP serves to

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mobilize retinol (vitamin A) from its stores in the liver and deliver it to extrahepatic tissues. Second, the interaction of RBP with TTR serves to prevent the glomerular filtration of the small RBP molecule and, hence, to reduce its renal catabolism.[2,5] In addition, there is evidence suggesting that the delivery of retinol to target tissues may involve the interaction of RBP with cell surface receptors.[6-8] Thus, RBP is involved in at least two, and possibly three, important physiologic interactions: with retinol, with TTR, and possibly with a cell surface receptor. From the x-ray crystallographic studies, a great deal is known about the ligand-binding domain of RBP.[4] However, very little is known about the region(s) of RBP involved in the RBP-TTR interaction or in the putative RBP-receptor interaction.

Monoclonal antibodies (MoAbs) are useful probes for the study of functional domains of proteins.[9-12] Accordingly, it is likely that MoAbs against human RBP would be valuable tools for investigating the various functional domains of RBP. In this study, we report the production and partial characterization of 12 MoAbs directed against human RBP, and the use of these 12 antibodies in studies designed to explore the antigenic determinants of RBP. The 12 antibodies were found to be directed against four distinct antigenic regions on RBP.

Materials and methods

Materials

Iscove's Modified Dulbecco's medium (IMDM), penicillin G, streptomycin sulphate, and fetal bovine serum were obtained from Gibco (Grand Island, NY, USA). Peroxidase-conjugated goat anti-mouse IgG + IgM was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Isotype-specific antisera were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL, USA).

Isolation of retinol-binding protein

The purified human RBP used for immunization was kindly provided by Dr. M. Kato of Shinshu University School of Medicine, Matsumoto, Japan. The RBP had been purified by methods similar to those previously reported from this laboratory for the isolation of human and rat RBPs.[1,13,14] The RBP preparation was homogeneous, as judged by migration as a single band of weight approximately 21 kd on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[15] The absorption spectrum showed the expected two peaks at 330 nm and 280 nm, with an absorbance ratio (A_{330}/A_{280}) of 0.80. Rabbit RBP was isolated from rabbit serum by repeated affinity chromatography on human TTR-linked to Sepharose 4B.[16] The rat RBP used in this study was isolated in this laboratory as described previously,[17] and had been stored at −20°C for about 10 years.

Immunization of mice and cell fusions

Male 8-week-old BALB/c mice (Charles River, Wilmington, MA, USA) were each given an intraperitoneal injection of 100 μg of purified human RBP emulsified in complete Freund's adjuvant. Four weeks later, each mouse received a booster intraperitoneal injection of 100 μg of purified human RBP emulsified in incomplete Freund's adjuvant. At least 4 weeks later, and 3 days prior to the fusion, each mouse received a second booster intraperitoneal injection of 100 μg of purified human RBP in saline.

Three separate fusions were performed according to the method of Pearson et al.[18] In brief, spleen cells (1.5×10^8) from immunized mice were fused with P3X63-Ag.8653 mouse myeloma cells (3.0×10^7), kindly provided by Dr. Elvin Kabat (Columbia University),[19] using 0.5 ml of 41.7% (wt/vol) polyethylene glycol (PEG)-15% (vol/vol) dimethyl sulfoxide (DMSO) in IMDM as the fusing agent. After the fusion, the cells were incubated at 37°C in IMDM supplemented with 20% (vol/vol) fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). The following day, the cells were plated in selective HAT medium[20] at a density of 2.5×10^5 spleen cells/well. After approximately 14 days, the wells were scored for hybrids and the media collected from over the growing hybrids were screened for the presence of anti-RBP antibody. At this time, the HAT medium was replaced by the same medium but without aminopterin (HT medium).

Screening of supernatant fluids of hybridomas

Supernatant fluids of hybridomas were tested for anti-human RBP antibody by an ELISA.[21] For each assay, aliquots of 50 μl of a solution of the purified human RBP that was used for immunization (20 μg/ml in 0.05 M carbonate buffer, pH 9.6) were distributed in wells of Immulon II plates (Dynatech Laboratories, Inc., Alexandria, VA, USA) and left overnight at 4°C. The following day, the wells were rinsed with phosphate-buffered saline (PBS; 0.01 M sodium phosphate, pH 7.2, 0.15 M NaCl) and nonspecific binding sites were saturated with 5% (wt/vol) bovine serum albumin (BSA) in PBS for 2 hours at 37°C. After washing the wells with PBS, 100 μl of hybridoma supernatant fluid was added to each well and incubated for 3 hours at 37°C. The ELISA reaction used peroxidase-conjugated goat anti-mouse IgG + IgM and a substrate solution containing o-phenylenediamine and hydrogen peroxide. Positive hybrids were transferred to larger culture vessels and were cloned twice by limiting dilution.[22] Selected subclones were injected intraperitoneally (1×10^7 cells/mouse) to pristane-treated BALB/c mice,[23] and the ascitic fluid was used as the source of antibody.

Purification of antibodies

Antibody isotypes were determined by double immunodiffusion.[24] Cell culture supernatants were pre-

cipitated with 50% ammonium sulfate at 4°C. The precipitates obtained were dialyzed extensively against PBS and then tested against isotype-specific antisera in 1% agarose plates.

IgG_1 subclass MoAbs were isolated from ascitic fluid by affinity chromatography on Protein A-Sepharose (Pharmacia, Piscataway, NJ, USA). IgA and IgM MoAbs were purified from ascitic fluid by precipitation with 50% ammonium sulfate followed by gel filtration on Sephacryl S-300.[26] The protein concentration of each purified antibody preparation was determined by the method of Bradford[27] using BSA as the standard.

Iodination of antibodies

Purified MoAbs were iodinated with Iodogen as described by Fraker and Speck.[28] Briefly, 40 μg of each antibody preparation was labeled with 2 mCi of Na[^{125}I] (New England Nuclear, Boston, MA, USA) to a specific activity of approximately 20,000 cpm/ng. Free ^{125}I was separated from the labeled antibody by chromatography on Sephadex G-50. The labeled antibodies were diluted and stored in 5% (wt/vol) BSA in PBS containing 0.01% (wt/vol) sodium azide. For each labeled antibody preparation, more than 92% of the radioactivity was precipitated with 10% (vol/vol) trichloroacetic acid. The labeled antibody preparations were used within a week after labeling.

Competitive binding assays

To determine whether the anti-RBP antibodies were directed against the same or different antigenic sites on RBP, solid-phase antibody competition radioassays were conducted.[29,30] Each MoAb was labeled with ^{125}I and made to compete with itself and with other unlabeled MoAbs for the binding to immobilized RBP. For each assay, Removawells II (Dynatech Laboratories, Inc., Alexandria, VA, USA) were coated with 50 μl of purified human RBP (20 μg/ml in 0.05 M carbonate buffer, pH 9.6) overnight at 4°C. Wells were then rinsed with PBS and nonspecific binding sites were saturated with 5% (wt/vol) BSA in PBS for 2 hours at 37°C. After rinsing with PBS, a series of wells received a constant amount (approximately 400,000 cpm/20 ng) of ^{125}I-labeled antibody together with increasing amounts of unlabeled antibodies (0 to 10^4 ng). After a 12-hour incubation at 23°C, the wells were rinsed with 0.05% Tween-20 in PBS, sliced, and radioassayed in a gamma counter. Nonspecific binding was calculated as the binding of the ^{125}I-labeled antibodies to BSA-coated wells. The results were expressed as $(B/B_o) \times 100$, where B = cpm bound minus nonspecific binding and B_o = cpm bound in the absence of competing antibody minus nonspecific binding. Triplicate determinations yielded coefficients of variation of less than 5%.

Gel electrophoresis and immunoblot analysis

SDS-PAGE was performed in 12.5% gels using a standard procedure.[15] Several samples of purified human RBP (approximately 5 μg/well) and of prestained protein standards (Bethesda Research Laboratories, Gaithersburg, MD, USA) were loaded on a gel. Once electrophoresis was completed, the proteins were transferred onto nitrocellulose as described by Towbin et al.[31] After transfer, the blots were cut in several strips, each containing a sample of human RBP and a sample of prestained protein standards. Each strip was then incubated for 4 hours at 23°C with conditioned media (10 ml) collected from over one of the different MoAb-producing hybridomas. The existence of specific antibodies directed against RBP (i.e., antibody-RBP interaction) was demonstrated using an immunoperoxidase staining technique.[32]

Immunoprecipitation of ^{125}I-labeled human retinol-binding protein

The reactivity and specificity of the anti-RBP MoAbs was also assessed by immunoprecipitation carried out by procedures similar to those described by Weisgraber et al.[9] For each assay, 100 μl of a solution of ^{125}I-labeled RBP (approximately 3.0×10^5 cpm) was incubated (12 hours at 4°C) with 100 μl of nonimmune mouse serum (1:1,000 dilution in PBS), 100 μl of 1% BSA-0.01% Tween-20 in PBS, and 100 μl of conditioned medium collected from each hybridoma. Antibody-bound ^{125}I-RBP was precipitated with goat anti-mouse IgG (5 μg/tube), then radioassayed in a gamma counter. Appropriate controls, omitting the hybridoma-conditioned media, were included. To verify that the anti-RBP antibodies were specifically immunoprecipitating RBP, some of the immunoprecipitated products were dissolved and subjected to SDS-PAGE on 12.5% slab gels.[15] After electrophoresis, the gel was fixed with 10% (vol/vol) trichloroacetic acid for 30 minutes with constant shaking, dried, and exposed to Kodak X-OMAT AR film at −70°C for 8 hours.

Reactivity of antibodies with human, rabbit, and rat retinol-binding proteins

To explore the epitopes of RBP recognized by these MoAbs, their ability to recognize human, rabbit, and rat RBPs was tested by immunoblotting. The amino acid substitutions between human, rabbit, and rat RBPs are known.[33] For each assay, approximately 5 μg of each species of RBP was subjected to SDS-PAGE, transferred onto nitrocellulose, and blotted as described above. The protein concentration of each RBP preparation was determined from absorbance at 280 nm using a value for $E^{1\%}$ of 19.4.[1]

Reactivity of antibodies with cyanogen bromide fragments of human retinol-binding protein

To try to localize the regions on RBP that react with these 12 anti-RBP MoAbs, their ability to recognize purified cyanogen bromide (CNBr) fragments of RBP was tested by a slot-immunobinding assay.[34] Retinol-binding protein contains four methionine residues; therefore, CNBr treatment should cleave the protein

into five fragments.[3,35] However, the Met 53-Ser 54 bond on RBP is relatively resistant to CNBr cleavage,[36] and hence, after CNBr digestion, only four fragments are often completely separated. These fragments are CNBr 1 (amino acid residues 1-27), CNBr 2 + CNBr 3 (amino acid residues 28-73), CNBr 4 (amino acid residues 74-88), and CNBr 5 (amino acid residues 89-102). The CNBr peptides of RBP were prepared and purified according to the method of Gawinowicz and Goodman.[37] Briefly, reduced and alkylated human RBP (approximately 2.8 mg) was dissolved in 0.5 ml of 70% formic acid and treated with 200-fold molar excess of CNBr. The CNBr fragments of RBP were isolated by HPLC in a reverse-phase C_{18}-μ Bondapak column (Waters Associates, Milford, MA, USA) using a linear gradient of 5-40% n-propanol containing 0.1% trifluoroacetic acid. The identity of each of the four CNBr fragments isolated was verified by its amino acid composition, kindly determined by Dr. M.A. Gawinowicz.[37]

For the slot-immunobinding assay, each CNBr fragment as well as intact purified human RBP (about 1 μg each) were dissolved in 50 μl of 0.05 M Tris-HCl, pH 6.8, containing 1% SDS and applied using a template to the nitrocellulose paper. After drying, the nitrocellulose membrane was handled as described above for immunoblotting the SDS-PAGE–separated proteins, with one exception: the ascitic fluid (1:20 dilution in PBS) from each hybridoma was used as the primary antibody.

Results

Cell fusions and monoclonal antibodies

Of the 2,000 wells that were used for the three fusions, growth was observed in 400 microtiter wells. Twenty-two hybridomas were identified by ELISA as positive for the secretion of anti-RBP antibody. Twelve of these hybridomas remained positive after transferring into larger culture vessels and cloning by limiting dilution. These clones were named 1A4, 1G10, 2F4 (first fusion); 5C5, 5H6, 5H9, 6C7, 6F4, 7G3 (second fusion); and 10G5, 13A1, 14E3 (third fusion). The rest of the clones either failed to grow or stopped producing antibodies.

Immunoblot analysis

To ensure that the 12 hybridomas selected by ELISA truly secreted specific antibodies against human RBP, their conditioned media were tested by immunoblot analysis. Hybridomas were plated at a density of 2.0×10^6 cells/100-mm culture dish and, after 60 hours in culture, their conditioned media were collected for testing. *Figure 1* shows the reactivity of the 12 hybridoma-conditioned media with purified human RBP. Each of the 12 MoAbs reacted strongly with SDS-denatured and blotted RBP. Treatment of holo-RBP with 2% SDS and 2.5% β-mercaptoethanol results in the dissociation of the ligand retinol from the RBP.

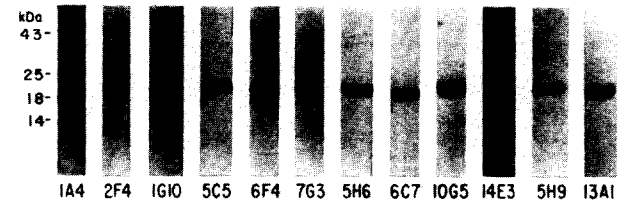

Figure 1 Testing the reactivity of the 12 MoAbs with human RBP by immunoblot analysis. Samples of purified human RBP (5 μg each) were subjected to SDS-PAGE and then transferred onto nitrocellulose sheets. After transfer, the sheets were cut into several strips that were treated to saturate nonspecific binding sites, then incubated with hybridoma conditioned media for 4 hours at 23°C. Bound antibody was detected immunoenzymatically as described in Materials and Methods.

This was verified by the absence of retinol fluorescence that could be observed in the RBP protein bands (migration at a size of approximately 21 kd) on illumination of the SDS-gel with ultraviolet light. Since all of the 12 MoAbs recognized SDS-treated and blotted RBP (*Figure 1*), this indicates that the antibodies not only recognized holo-RBP (as determined by ELISA), but also apo-RBP.

Immunoglobulin chain class and subclass

Each of the 12 antibodies obtained reacted with only one of the isotype-specific antisera used in the double immunodiffusion assays, indicating their true monoclonality. The MoAbs were found to be of three different isotypes: IgG1 (antibodies 1G10, 5C5, 6F4, 7G3, 10G5, 14E3, 5H9, and 13A1), IgM (1A4, 2F4, and 5H6), and IgA (antibody 6C7). Although IgE- and IgA-secreting hybridomas are rarely obtained, IgA-secreting cells have been identified in spleens of immunized mice.[38] One of the 12 anti-human RBP antibodies obtained was of this isotype.

These clones were injected intraperitoneally into syngeneic mice, and the ascitic fluid obtained was used as a source of antibody. The yield of purified MoAb from 1 ml of ascites varied from 1.5 to 3.5 mg.

Immunoprecipitation of ^{125}I-labeled human retinol-binding protein

Seven of the eight IgG anti-RBP MoAbs were also tested for their ability to immunoprecipitate ^{125}I-labeled human RBP. (The hybridoma line 1G10 was not tested because it had been frozen at the time this assay was done.) Monoclonal antibodies 5C5 and 7G3 immunoprecipitated 85% and 95%, respectively, of the total counts. Antibodies 5H9, 6F4, 10G5, 13A1, and 14E3 each immunoprecipitated 16% to 25% of the total counts added. In several instances (antibodies 5C5, 5H9, and 7G3), the immunoprecipitation products were solubilized and analyzed by SDS-PAGE. In each case, a single labeled protein band corresponding to RBP (weight, approximately 21 kd) was seen (data not shown in detail).

Table 1 Cross-competition between anti-human retinol-binding protein antibodies

Competitor antibody	Radiolabeled antibody											
	1A4	2F4	1G10	5C5	6F4	7G3	5H6	6C7	10G5	14E3	5H9	13A1
1A4	3+	3+	±	±	—	—	—	—	±	—	1+	±
2F4	3+	3+	1+	1+	—	—	—	—	—	±	1+	1+
1G10	—	—	3+	2+	2+	3+	—	—	±	—	3+	3+
5C5	±	±	3+	3+	3+	3+	—	—	—	±	3+	3+
6F4	±	±	3+	3+	3+	3+	—	—	—	—	3+	3+
7G3	—	±	3+	3+	3+	3+	—	—	—	—	3+	3+
5H6	—	±	—	ND	ND	ND	3+	ND	3+	ND	3+	3+
6C7	—	—	—	—	—	—	3+	3+	3+	3+	3+	3+
10G5	—	—	—	±	—	ND	2+	3+	3+	ND	3+	3+
14E3	—	±	±	—	—	±	2+	3+	3+	3+	3+	3+
5H9	±	ND	ND	ND	ND	ND	ND	ND	ND	ND	3+	ND
13A1	1+	1+	3+	3+	2+	2+	1+	1+	2+	2+	3+	3+

Solid phase antibody competition assays. Microtiter wells were each coated with 50 μl of purified human RBP (20 μg/ml), then received increasing amounts (0 to 10^4 ng) of unlabeled antibodies and a constant amount of labeled antibody. Results are expressed according to the extent of the competition at the highest level of competing antibody studied: 3+ = > 80% displacement of B_0 counts; 2+ = 60% to 80%; 1+ = 40% to 60%; ± = 20% to 40%; — = < 20%. ND = not determined. B_0 counts = labeled antibody bound in the absence of unlabeled antibody.

Antibody competition for the binding to human retinol-binding protein

To assess whether the 12 anti-RBP MoAbs bound to the same or different epitopes on RBP, each antibody was allowed to compete with the other antibodies for binding to RBP. The results of the antibody cross-competition studies are summarized in *Table 1*. (Complete data for four of the antibodies are presented below. It should be emphasized that the interpretation of the cross-competition studies was made by examination of the complete displacement curve patterns, for each of the 12 labeled antibodies, and not only on the basis of the selected data summarized in *Table 1*.) Since the ascites tumors of antibodies 5H6, 5H9, and 10G5 grew very slowly, these three antibodies were not used as competitors in the binding studies of all of the labeled antibodies. Each of the ^{125}I-labeled antibodies was effectively displaced from the binding to RBP by the corresponding unlabeled antibody, as expected for specific antibody binding.

Antibodies 1A4 and 2F4 competed strongly against each other, whether binding to RBP of labeled 1A4 or of labeled 2F4 was tested (*Figure 2A*). The other antibodies showed either no effect, or only weakly inhibited the binding of labeled 1A4 and 2F4 to RBP. Since antibodies 1A4 and 2F4 displaced each other from the binding to RBP, the epitopes of RBP recognized by these two antibodies appeared to be either the same or very close to each other.

Antibodies 1G10, 5C5, 6F4, and 7G3 competed strongly against each other. Although the strength and pattern of cross-competition differed among these four antibodies (*Figure 2B*), in all cases, binding was decreased by competition with each of the unlabeled antibodies at the highest levels tested by at least 75%. Antibody 13A1 also decreased the binding of 1G10, 5C5, 6F4, and 7G3 to RBP, whereas the other antibodies tested did not affect or only weakly inhibited the binding of these four labeled antibodies to RBP. Because antibodies 1G10, 5C5, 6F4, and 7G3 displayed strong mutual competition for the binding to RBP, the antigenic determinants recognized by these antibodies appeared to be the same or very closely associated on the RBP molecule.

Antibodies 5H6, 6C7, 10G5, and 14E3 showed strong mutual competition for the binding to RBP as well as similar patterns of competition with the rest of the antibodies. Here, too, the strength and pattern of cross-competition differed among these four antibodies (*Figure 2C*). The binding of these four antibodies was also significantly decreased by antibody 13A1. The other antibodies showed a slight inhibitory effect on the binding of labeled antibody to RBP or no inhibition at all. Because antibodies 5H6, 6C7, 10G5, and 14E3 competed strongly against each other, it appeared that these four antibodies recognized the same or closely associated epitopes on RBP.

The binding of labeled antibody 5H9 to RBP was almost completely (90%) blocked by unlabeled antibody 13A1. Although the reverse experiment was not carried out, the binding of both of these labeled antibodies to RBP was markedly reduced (at least 75%) by antibodies 1G10, 5C5, 6F4, 7G3, 5H6, 6C7, 10G5, and 14E3. Antibodies 1A4 and 2F4 also diminished by about 40% the binding of 5H9 and 13A1 to RBP. These results suggest that antibodies 5H9 and 13A1 recognize the same or closely associated sites on RBP.

In summary, the results of the antibody cross-competition radioassays demonstrated the presence of four distinctive and different patterns of antibody cross-competition. On the basis of these patterns, the 12 MoAbs could be classified as belonging to four distinct groups, which we called groups I, II, III, and IV. Representative competitive displacement curves from each group of antibodies are shown in *Figure 2*. Group I antibodies (1A4 and 2F4) are represented by competition with labeled antibody 2F4 in panel A. Group II antibodies (1G10, 5C5, 6F4, and 7G3) are represented

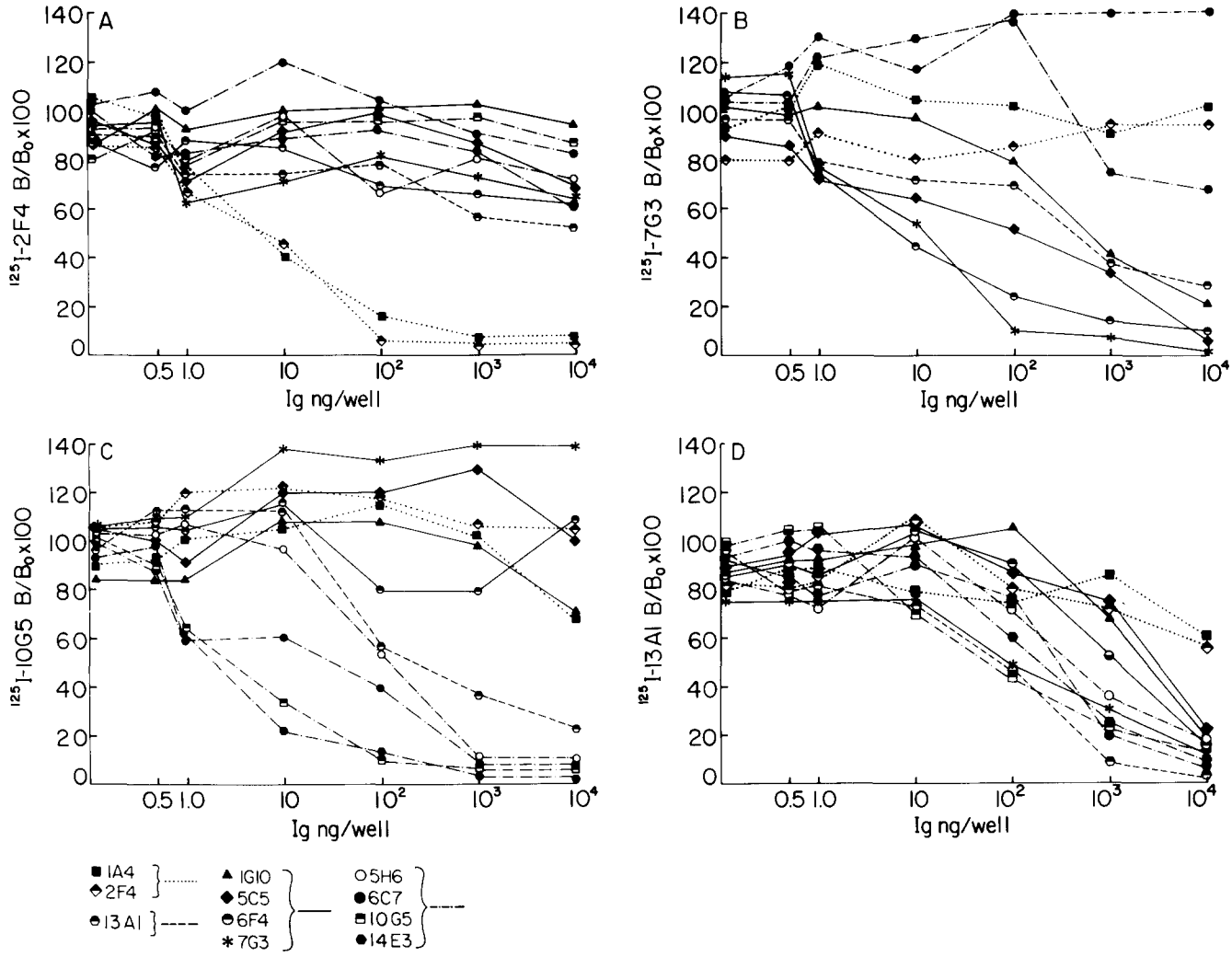

Figure 2 Representative antibody cross-competition experiments. (A) Group I antibodies represented by 2F4; (B) group II antibodies represented by 7G3; (C) group III antibodies represented by 10G5; and (D) group IV antibodies represented by 13A1. Microtiter wells were each coated with 50 μl of purified human RBP (20 μg/ml) overnight at 4°C and rinsed with PBS; nonspecific binding sites were saturated with BSA. Wells then received increasing amounts (0 to 10^4 ng) of unlabeled antibodies and a constant amount of ^{125}I-labeled antibody. After incubation, the wells were rinsed and counted.

by competition with labeled antibody 7G3 in panel B. Group III antibodies (5H6, 6C7, 10G5, and 14E3) are represented by competition with labeled antibody 10G5 in panel C. Group IV antibodies (5H9 and 13A1) are represented in panel D by competition with labeled antibody 13A1. Since the pattern of antibody cross-competition of each of these four groups of antibodies is different from the patterns of the other three groups, we conclude that each of these groups of antibodies recognizes a different and distinct antigenic region on RBP.

Although antibodies within a group showed strong mutual competition, and were hence classified together, as already indicated above, in several instances there were significant differences in their displacement curves. This is clearly shown in *Figure 2B* and *2C* for antibodies classified within groups II and III, respectively. The groupings are clearly not homogenous. This observation suggests that MoAbs within a group, in both groups II and III antibodies, recognize a set of closely associated epitopes rather than the same epitope within a single region of the molecule. Thus, these results indicate that at least four regions of the RBP molecule can serve as antigenic determinants, and that different closely associated epitopes can exist within a given antigenic domain.

Reactivity of antibodies with human, rabbit, and rat retinol-binding proteins

The reactivity of each antibody with human, rabbit, and rat RBPs was tested by immunoblotting. A comparison of the primary structures of human, rabbit, and rat RBPs has been reported by Sundelin et al.[33] As reported,[33] some amino acid sequences are totally conserved between the three species. Other regions are only conserved between human and rabbit RBP, whereas other regions show considerable interspecies differences.

When each of these 12 MoAbs was tested with hu-

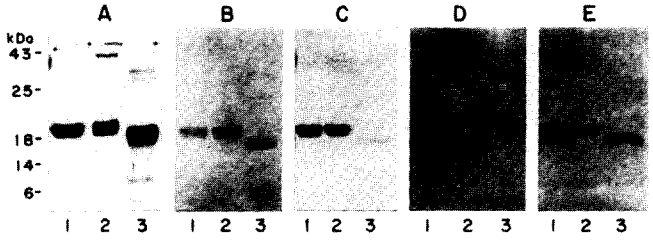

Figure 3 Testing the reactivity of anti-human RBP MoAbs by immunoblot analysis with human, rabbit, and rat RBPs. About 5 μg of purified human (1), rabbit (2), and rat (3) RBPs were subjected to SDS-PAGE and transferred onto nitrocellulose sheets. The nitrocellulose sheets were incubated with hybridoma-conditioned media and bound antibody was detected immunoenzymatically, as described in Materials and Methods. (A) Coomassie Blue-stained gel, (B) group I antibodies represented by 2F4, (C) group II antibodies represented by 7G3, (D) group III antibodies represented by 14E3, and (E) group IV antibodies represented by 13A1.

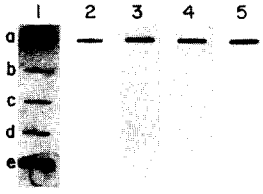

Figure 4 Testing the reactivity of anti-RBP antibodies with CNBr fragments of human RBP. About 1 μg each of native human RBP (sample a), CNBr 1 (sample b), CNBr 2 + CNBr 3 (sample c), CNBr 4 (sample d), and CNBr 5 (sample e) was spotted onto nitrocellulose sheets. The nitrocellulose sheets were incubated with ascitic fluid (1:20 dilution in PBS) and bound antibody was detected immunoenzymatically. (1) Polyclonal anti-human RBP antiserum (1:1,000 dilution), (2) group I antibodies represented by 2F4, (3) group II antibodies represented by 7G3, (4) group III antibodies represented by 14E3, and (5) group IV antibodies represented by 13A1.

man, rabbit, and rat RBPs, three distinct patterns of reactivity were observed. *Figure 3* shows a representation of the patterns observed. Group I antibodies (1A4 and 2F4), which are represented by antibody 2F4 in panel B, reacted to a similar extent with RBP from all three species (i.e., with human, rabbit, and rat RBPs). Group II antibodies (1G10, 5C5, 6F4, and 7G3), which are represented by antibody 7G3 in panel C, reacted strongly with human and rabbit RBPs but reacted very weakly with rat RBP. In contrast, group III (5H6, 6C7, 10G5, and 14E3) antibodies, which are represented by antibody 14E3 in panel D, reacted strongly with human RBP but did not react with rabbit or rat RBP. Similar to group I antibodies, group IV antibodies (5H9 and 13A1), which are represented by antibody 13A1 in panel E, reacted with comparable strength with human, rabbit, and rat RBPs.

Reactivity of antibodies with cyanogen bromide fragments of retinol-binding protein

A preliminary study was conducted to try to localize the epitopes recognized by these MoAbs. In this study, the reactivity of each antibody with the four CNBr fragments of human RBP (CNBr 1, residues 1-27; CNBr 2 + CNBr 3, residues 28-73; CNBr 4, residues 74-88; and CNBr 5, residues 89-182) was tested by a slot immunobinding assay. A polyclonal anti-human RBP antiserum (1:1,000 dilution in 1% BSA in PBS) was used as a positive control. As shown in *Figure 4*, the polyclonal antiserum (panel 1) reacted strongly with intact RBP (sample a); it also reacted with each of the four purified CNBr fragments of RBP (samples b, c, d, and e), and particularly strongly with CNBr 5 (the largest of the CNBr fragments). All of the 12 MoAbs also reacted with intact RBP (sample a; panels 2 through 5). However, in contrast with the polyclonal antiserum, none of the MoAbs reacted with any of the CNBr fragments (panels 2 through 5; samples b, c, d, and e).

Discussion

This report describes the production and partial characterization of a battery of MoAbs directed against human RBP. The primary objective of this work was to explore the antigenic determinants on the RBP molecule, using newly produced MoAbs directed at different epitopes on RBP. The present study represents the first phase of a project that aims, as its long-term goal, to use these MoAbs to study and map the functional domains of RBP.

Twelve MoAbs directed against human RBP were produced and partially characterized. The antibodies were obtained from three separate cell fusions which used spleen cells of mice immunized with purified human RBP. The 12 antibodies reacted with holo-RBP in ELISAs, as well as with apo-RBP by Western blotting after SDS-PAGE. Thus, the antigenic sites recognized by these antibodies were not masked by the change in protein conformation that occurs after treatment with SDS.[39,40] The anti-RBP MoAbs also reacted with human RBP in solution, and could be used to immunoprecipitate labeled RBP. The differences in reactivity of the different MoAbs with labeled human RBP in the immunoprecipitation assay were probably due to combined effects of differences in concentration of antibody per milliliter of conditioned medium, and differences in antibody affinities.

A solid-phase antibody competition assay was used to determine which antibodies competed with one another for binding to RBP. These results provide information about the number of different antigenic sites recognized by the different antibodies and about their spatial proximity. Based on the antibody cross-competition studies, the 12 anti-RBP antibodies were classified into four major groups. Each of these groups of antibodies displayed a distinctive and different pattern of antibody cross-competition (*Figure 2*). Hence, the different MoAbs appear to be directed at four distinct antigenic regions on RBP, and it can be concluded that RBP contains at least four major antigenic domains. Although antibodies within each group competed strongly against each other, in several cases there were significant differences in their displacement curves (*Figure 2B* and *2C*). Thus, the antibodies classi-

fied within a group, particularly in groups II and III, appear to recognize a set of closely associated epitopes, rather than all recognizing the same epitope, in a single region of the molecule. In addition, the cross-competition studies provided some information about the spatial proximity of the epitopes of the four different groups of antibodies. Antibodies of groups I, II, and III showed no intergroup competition or only weak intergroup competition. These results indicate that the groups I, II, and III antibodies recognize regions of the RBP molecule that are not only different but physically distinct or separate from each other. On the other hand, group IV antibodies competed strongly with group II and group III antibodies. This suggests that group IV antibodies are directed at epitope(s) that lie between, and/or are in close proximity to, the epitopes of the group II and group III antibodies. Similar relationships between antibodies were observed by Milne et al.[41] in studies of MoAbs against apolipoprotein B (apoB). Milne et al.[41] observed antibodies that competed strongly with each other, antibodies that did not compete against each other, and antibodies that hindered the binding of two different antibodies.

To obtain additional information about the epitopes of RBP recognized by these MoAbs, the reactivity of each antibody with human, rabbit, and rat RBPs was tested. The primary structures of human, rabbit, and rat RBPs are known, and the conserved and nonconserved regions across these three species of RBP have been defined.[33] This approach of comparing the reactivities of MoAbs with antigens from different animal species has been used successfully by others for the classification of MoAbs according to their specificities for conserved and nonconserved regions of antigens.[9,42,43] When the reactivity of our antibodies with human, rabbit, and rat RBPs was tested, three different patterns of reactivity were observed. Group I and group IV antibodies reacted to a similar extent with RBP from all three species (i.e., human, rabbit, and rat RBPs), indicating reactivity with regions of RBP that are conserved across the three species. The conserved regions of RBP have been identified as amino acid residues 1-20, 69-98, and 126-141.[33] Group II antibodies reacted strongly with human and rabbit RBP, but very weakly with rat RBP, suggesting that group II antibodies recognize regions that are conserved between human and rabbit RBPs but not in rat RBP; such regions include residues 22-49, 53-68, 115-124, and 163-174.[33] In contrast, group III antibodies only recognized human RBP, indicating reactivity with nonconserved regions of RBP; such regions include amino acid residues 145-151 and 175-179.[33] The results of this study support our classification of the 12 MoAbs into four groups that are directed at different and distinct regions of the RBP molecule.

Since the mouse and the rat are closely related species, it is of interest that four of the 12 anti-human RBP mouse MoAbs produced in this study reacted strongly with rat RBP. Monoclonal antibodies that recognize conserved regions of proteins have been found to be valuable probes for the characterization of functional domains of proteins.[9,42,43] For example, Young et al.[42] reported that an anti-human apoB MoAb that recognized apoB from a variety of other species, such as dog, pig, guinea pig, and rabbit, was able to block the binding of ^{125}I-labeled low-density lipoprotein (LDL) to cultured fibroblasts. By mapping the epitopes of this and of four other anti-apoB antibodies that also blocked the cellular uptake of LDL,[41] the amino acid residues on apoB involved in mediating receptor binding have been partially identified.[12] Similarly, Weisgraber et al.[9] demonstrated that an anti-human apoE MoAb that blocked the binding of human apolipoprotein E (apoE) containing lipoproteins to fibroblasts was also able to bind with low affinity to canine apoE. Hence, group I and group IV anti-RBP antibodies which recognize human, rabbit, and rat RBPs might prove particularly valuable for the study of the conserved functional domains of RBP.

In a preliminary experiment that attempted to localize the epitopes recognized by these antibodies, the reactivity of each antibody with CNBr fragments of RBP was explored. Under the conditions used, none of the MoAbs recognized any of the isolated CNBr fragments of RBP. More extensive studies of this issue will need to be conducted in the future. A lack of reactivity of the MoAbs with the CNBr fragments suggests that either the epitopes of these antibodies are conformation dependent or that the epitopes of these antibodies are close to the methionine residues at which CNBr cleaves. It is not uncommon that MoAbs fail to react with CNBr fragments of a protein. For example, Collins et al.[44] reported that of 26 MoAbs produced against human TTR, none reacted with the CNBr fragments of TTR. Likewise, Berzofsky et al.[45] reported that of six MoAbs raised against sperm whale myoglobin, none reacted with any of the three CNBr fragments of myglobin despite the fact that a polyclonal mouse antiserum did. Therefore, other methods will be needed to more specifically identify the location of the epitope for each antibody on the RBP molecule.

Retinol-binding protein interacts with its ligand (retinol), with TTR, and possibly with a cell surface receptor. From the x-ray crystollographic study of holo-RBP,[4] much is known about the molecular interaction of RBP with retinol. Although there is strong evidence that the region(s) of RBP involved in the RBP-TTR interaction have been conserved through evolution,[2,46] at present, the amino acids on RBP involved in the binding site with TTR have not been identified. Very little is known about the interaction of RBP with its putative cell surface receptor. In the future, the MoAbs described here might be useful in studies designed to explore the domains of RBP involved in its interaction with TTR, and possibly with a cell surface receptor.

The MoAbs described in this study of the antigenic determinants of RBP also might be useful in studies of the family of proteins structurally homologous to RBP that bind small hydrophobic molecules.[47] The members of this protein family include RBP, β-lactoglobulin, apolipoprotein D, α_{2u}-microglobulin,

odorant-binding protein, insect bilin-binding protein (insecticyanin), α_1-microglobulin, and androgen-dependent secretory protein.[47-53] These proteins all display a moderate degree of amino acid sequence homology with each other. Several of these proteins have the function of binding, and possibly transporting, hydrophobic ligands; the function of some of the proteins in the family is not known. The structures of three of these proteins (RBP,[4] β-lactoglobulin,[48,49] and bilin-binding protein[50-52]) have been determined by x-ray crystallography and found to be remarkably similar. It seems likely that all of the proteins in this family will be found to have similar three-dimensional structures and that they all function to bind hydrophobic ligands. Hence, it would be interesting to explore the structural significance of conserved versus non-conserved regions. The MoAbs described in this report might be valuable reagents for such studies.

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