# BINDING PROPERTIES OF IMMUNOGLOBULIN COMBINING SITES SPECIFIC FOR TERMINAL OR NONTERMINAL ANTIGENIC DETERMINANTS IN DEXTRAN*

By JOHN CISAR,‡ ELVIN A. KABAT, MARIANNE M. DORNER,§ AND JERRY LIAO

*(From the Departments of Microbiology, Human Genetics and Development, and Neurology, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, Presbyterian Hospital, New York 10032)*

Since the discovery by Landsteiner (1, 2) that low molecular weight haptens would react with antihapten antibody to inhibit precipitation by azoprotein antigens, and of Marrack and Smith (3) that binding of haptens to antibody could be demonstrated by equilibrium dialysis, such interactions have been used for identifying antigenic determinants and for establishing specificities and sizes of antibody combining sites (cf. 4, 5).

Specificities for carbohydrate antigens often appear to be directed toward the structure and linkage of terminal nonreducing sugars. This was first recognized by Goebel, Avery, and Babers (6), who found that the structure of the terminal nonreducing sugar was of predominant importance in determining cross-reactions with rabbit antisera to various disaccharides conjugated to horse serum globulin. Later, Karush (7) showed that lactoside-specific rabbit antibodies, produced by immunization with a lactosyl-azoprotein conjugate, had the major portion of their binding energies directed against the terminal $\beta$-linked galactose. With glycoproteins and polysaccharides there are many examples of terminal antigenic determinants. These include the A, B, H, and Lewis specificities of human blood group substances (8, 9), terminal glucuronic acid residues which are involved in the specificity of Type II pneumoccous polysaccharide (10–13 cf. 4, 5, 14), and terminal $\beta$-*N*-acetylglucosamine for Group A (15) and $\alpha$-*N*-acetylgalactosamine for Group C (16) specificities in streptococcal polysaccharides (cf. 17). Certain determinants in teichoic acids (17–21 cf. 22) and in the somatic antigens of *Salmonella* (23, 24) and *Shigella* (25) are also of this type.

Antigenic determinants which do not require a terminal end group have also been recognized. Polysaccharides such as the type III and VIII pneumococcal polysaccharides which were shown to be linear (26) and thus to have one nonreducing end per milecule had long been known to precipitate with homologous antisera and also to cross-react (cf. 4). The determinant of the Type III polysaccharide (S III) was shown to consist of three repeating units of the disaccharide, cellobiuronic acid (27). Moreover, Heidelberger and

* Aided by a grant from the National Science Foundation GB 35243-X-1, X-2 and BMS-72-02219-A02, and in part by a General Research Support Grant from the United States Public Health Service to Columbia University.

‡ Present address: School of Dentistry, University of Colorado Medical Center, Denver, Colorado 80220.

§ Present address: Medizinische Universitaets-Klinik Bergheimer Strasse 58, 6900 Heidelberg, Germany.

Rebers (28) attributed the cross-reactivity between anti-S IV sera and S II to (1 → 3)-linked L-rhamnose units which occur at intervals along linear sugar chains. Nonterminal determinants are important in *Shigella* (25) and *Salmonella* (24), examples being determinants 3 and 15 of group E *Salmonella* which involve repeating [$\beta$ D Gal(1→6)D Man-(1→4)L Rham(1→3)] sequences (29). In addition, certain specificities for teichoic acids (30 cf. 22) are of this type. It has been suggested that steric factors play a role in the proper exposure of certain terminal (31–33) and nonterminal (34) sugar determinants.

Dextrans are branched polymers of $\alpha$-linked D glucopyranosyl units and their relatively simple structures have made them especially useful in immunochemical studies of homopolysaccharide antigens. Specificities involving $\alpha(1 \rightarrow 6)$- and $\alpha(1 \rightarrow 2)$-, $\alpha(1 \rightarrow 3)$-, $\alpha(1 \rightarrow 4)$-linked D glucopyranosyl units have been described (35-43). The reactions of $\alpha(1 \rightarrow 6)$-specific antidextrans have been thought to occur at the nonreducing ends of glucose chains with the terminal $\alpha(1 \rightarrow 6)$-linked D glucopyranosyl residue being immunodominant. The evidence for this has come from the structure of these polysaccharides which have several nonreducing ends but only one reducing end, and from inhibition of precipitation studies with isomaltose oligosaccharides from which it appeared that a majority of the binding energy was contributed by the terminal nonreducing glucose (44, 45, cf. 4, 5). In addition, rabbits which had been immunized with bovine serum albumin (BSA)[1] conjugates of isomaltotrionic acid (IM3-CONH-BSA) (46), and isomaltohexaonic acid (IM6-CONH-BSA) (47), produced antibodies whose quantitative precipitin reactions with dextrans were similar to those of $\alpha(1 \rightarrow 6)$-specific antidextrans. However, the best inhibitor for certain rabbit sera to IM3-CONH-BSA was larger than the trisaccharide which suggested as one possibility that specificities for nonterminal antigenic determinants such as (-*O*-(1 → 6)-D Glc-$\alpha$-*O*-) sequences might exist (46). Moreover, Richter (48) has shown that rabbit antibodies to a dextran-protein conjugate made with a dextran fragment of mol wt 4,400 could precipitate a synthetic linear dextran (49, 50) which implied that these antibodies were capable of reacting with nonterminal glucosyl residues. Concanavalin A, which is thought to react at terminal nonreducing ends of dextran chains, does not precipitate with synthetic linear dextran (51). These findings raise two questions of fundamental importance: (*a*) Are antidextrans and antibodies against isomaltosyl oligosaccharides coupled to protein, mixtures of antibodies with specificities directed toward terminal nonreducing as well as nonterminal oligosaccharide sequences? (*b*) Can antibodies against terminal determinants cross-react at nonterminal locations along the dextran chain?

The existence of several homogeneous BALB/c mouse myeloma proteins with specificities for dextrans (52–55), fructosans (54–56), and galactans (57–59) provides a very useful approach to these questions. Three dextran-reactive myeloma globulins, W3129, W3434 and QUPC 52, have been shown (55) to have $\alpha(1 \rightarrow 6)$ specificities. Proteins W3129 and W3434 differed slightly in specificity (55) and idiotype (60), but both showed maximum complementarity to isomaltopentaose (IM5) while protein QUPC 52 showed maximum binding for isomaltohexaose (IM6) and was not related idiotypically to W3129.

We have now determined the binding energies for the interactions of proteins W3129 and QUPC 52 with each member of the isomaltose series and with methyl $\alpha$Dglucoside. These results indicate the protein W3129 has a nonreducing terminal specificity for $\alpha(1 \rightarrow 6)$ chains of dextran while protein QUPC 52 has nonterminal specificity. This was confirmed by precipitin reactions with a synthetic linear dextran which failed to precipitate protein W3129 but precipitated QUPC 52. The correlation between the binding properties of these myeloma proteins and their precipitin reactions with the synthetic dextran (49, 50) provides further evidence that this dextran reacts immunochemically as a

completely linear molecule. In addition, quantitative precipitin studies with linear and branched dextrans favor the concept that some human antidextran molecules also react only at terminal nonreducing ends while others can react at nonterminal locations along linear dextran chains. Moreover, rabbit antisera against IM3 or IM6 coupled to BSA[1] contain fractions of molecules which precipitate linear dextran and thus must be capable of nonterminal reactions with dextran chains. These findings provide a fundamental insight into the immunochemistry of dextran and probably other polysaccharide antigens.

## Materials and Methods

*Human and Rabbit Antisera and Mouse Myeloma Proteins.* Human antidextrans are listed in Table I along with the dextrans employed for immunization. Serum 1 $D_{54-60}$ was a pool obtained by plasmapheresis of subject 1 seven times over a period of 2 mo, and was taken 18½ years after immunization with dextran B1255. Rabbit antisera against IM3-CONH-BSA (46) and IM6-CONH-BSA (47) were those studied previously. BALB/c mouse IgA myeloma proteins W3129, QUPC 52, W3082, and UPC 61 have been described (55) and were from serum or ascites fluid generously provided by Doctors Melvin Cohn and Martin Weigert (The Salk Institute, San Diego, Calif.), and Dr. Michael Potter of NIH.

*Purification of Human Antidextrans and Mouse Myeloma Proteins.* Purification of antidextrans and myeloma proteins was by a standard batch-wise immunoadsorption procedure (63–65) using Sephadex G75 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) for human antidextrans and proteins W3129 and QUPC 52 and levan gel (66, 68, 69) for human antilevans and proteins W3082 and UPC 61. After adsorption of protein, the insoluble gels were washed with 0.01 M phosphate-buffered saline, pH 7.2, 0.02% sodium azide (PBS) until supernates gave negligible absorption at 280 nm. Hapten elution was at 37°C for 1 h and proteins were freed of hapten by at least two passes through columns of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) (68, 69) and concentrated by ultrafiltration with collodion bags (Schleicher & Schuell, Inc., Keene, N. H.) or above a Diaflow UM-10 membrane (Amicon Corporation, Lexington, Mass.).

Antidextrans from 3,726 ml of 1 $D_{54-60}$ which contained 15.1 mg antidextran nitrogen (N) were adsorbed on to 922 mg Sephadex G75 and eluted with IM3 and IM6 in a batch-wise modification of the procedure described by Harisdangkul and Kabat (68). The packed Sephadex G75 was eluted first with 270 mg of IM3 in 15 ml PBS. This eluate (1 $D_{54-60}$ IM3El 1) was concentrated and the ultrafiltrate containing most of the IM3 was used for the second IM3 elution and after this, the process was repeated a third time. The Sephadex was then washed with PBS and the washings were combined with the second and third IM3 eluates to give 1 $D_{54-60}$ IM3El 2–3. Then 202 mg IM3 in 15 ml PBS were added to the packed Sephadex and this eluate plus the next two were combined to give 1 $D_{54-60}$ IM3El 4–6. The washed Sephadex was then extracted six times as above starting with 90 mg IM6 in 15 ml PBS and these were pooled to give 1 $D_{54-60}$ IM6El 1–6. The total recovery from all elutions was 10.2 mg antidextran N (68%) of which 1 $D_{54-60}$ IM3El 1, El 2–3, El 4–6, and IM6El 1–6 accounted for 52, 21, 8, and 19% respectively. After removal of antidextrans, human antilevans were purified from 1 $D_{54-60}$ sera by elution from levan gel with 0.1 M acetate buffer, pH 3.7 (66, 68).

Dextran- and fructosan-specific mouse myeloma proteins were eluted with methyl αDglucoside and sucrose respectively, which had previously been dialyzed to remove polysaccharides (70) and recrystallized from ethanol. With protein W3129, 45.5 mg of myeloma N was adsorbed to 4 g of Sephadex G75, and eluted three times with about 200 mg methyl α D glucoside per mg of myeloma N; the final recovery was 36 mg W3129 N (79%). With protein QUPC 52, 92 mg of myeloma N was adsorbed to 6.3 g of Sephadex, washed, and eluted 10 times with methyl αDglucoside at about 420 mg methyl αDglucoside per mg of myeloma N; the final recovery was only 26 mg QUPC 52 N (28%). The G75 was then eluted repeatedly in the cold (30 min per elution) with 0.1 M glycine HCl buffers ranging from pH 3.0 to pH 1.8 and eluates were neutralized immediately after collection. The

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; IM2, isomaltose; IM3, isomaltotriose; IM4, isomaltotetraose; IM5, isomaltopentaose; IM6, isomaltohexaose; IM7, isomaltoheptaose; PBS, phosphate-buffered saline.

TABLE I
*Human Antidextrans*

| Antidextran | Immunizing dextran | Linkages | | | Reference |
|---|---|---|---|---|---|
| | | $\alpha(1\rightarrow6)$ | $\alpha(1\rightarrow3)$-like | $\alpha(1\rightarrow4)$-like | |
| | | % | % | % | |
| $1D_{54\text{-}60}$ | B1255 (Native) | 86 | 0 | 14 | 4, 5, 36, 37, 45, 61, 63, 65, 66, 67, 68 |
| $20D_{10}$ | OP 155 (Clinical) | 88 | | | 4, 5, 36, 44, 45, 61, 63, 64, 65, 66, 67 |
| $30D_3$ | OP 163 (Clinical) | 88 | | | 4, 5, 35, 36, 37, 44, 45, 61, 66, 67 |
| $116D_1$ | S-5-A-1.0 (Clinical) | 86 | 10 | 4 | 4, 37 |
| $176D_4$ | NRC Fr. 4 (Clinical) | 96 | 4 | 0 | 4, 5, 45, 62, 64 |
| $219D_3$ | APC-54 (Clinical) | | | | 62 |

final yields were: 2.4 mg N at pH 3.0 (one elution) 11.8 mg N at pH at pH 2.7 to 2.4 (four elutions), 13.7 mg N at pH 2.0 (three elutions), and 4.6 mg N at pH 1.8 (three elutions) giving a total recovery in the low pH elutions of 32.5 mg QUPC 52 N (35%). All eluates were between 90 and 95% reactive with Sephadex G75. With proteins W3082 and UPC 61, 1.2 mg myeloma N was adsorbed onto 65 mg levan gel and eluted twice with 300 mg sucrose per mg myeloma N which removed virtually all adsorbed protein.

*Dextrans.* Native dextran B512 and clinical dextran N-150N have been described (61 cf. 4). Prof. Conrad Schuerch, Chemistry Department, State University College of Forestry, Syracuse, N. Y., kindly provided dextran D3 which by chemical methods appears to be a completely linear polymer of $\alpha$ (1 → 6)-linked D glucopyranosyl units having a mol wt by viscosity of 36,500 (49, 50). Enzymatic degradation experiments indicate that synthetic dextran contains 1–2% of structural flaws (71).

*Qligosaccharides.* The isomaltose oligosaccharides, methyl $\alpha$D-glucopyranoside, the $\beta$ (2 → 1)-linked series of fructose oligosaccharides and sucrose were those described (55). Methyl $\alpha$-isomaltoside and methyl $\alpha$-isomaltotrioside (72) were provided by Dr. Allene Jeanes and have been studied previously (4, 65).

Tritium-labeled isomaltoheptitol ([$^3$H]IM7-OH) was prepared by overnight reduction at 4°C of 90 $\mu$mol IM7 with 25 $\mu$mol [$^3$H] sodium borohydride (383 mCi/nmol), and the reaction was stopped by adding small amounts of 25% acetic acid. The partially reduced and tritiated IM7 was then completely reduced with a 20-fold molar excess of $NaBH_4$. After 2 days at 4°C, excess $NaBH_4$ was destroyed and the [$^3$H]IM7-OH was purified by ethanol elution from a charcoal-celite column, followed by preparative paper chromatography with a propanol-ethanol-water (6:1:3) solvent system and final chromatography on a Bio-Gel P-2 column (minus 400 mesh, 70 × 1.9 cm) (73).

*Immunochemical Methods.* Quantitative precipitin and inhibition assays were done by a microtechnique (4) and total N in the washed precipitates was measured by the ninhydrin method (74). The separation of IgA monomer and polymer fractions of protein QUPC 52 and quantitative immunoadsorption experiments with dextran-reactive proteins using Sephadex G75 were like those described for protein W3129 (55).

*Isoelectric Focusing.* Analytical isoelectric focusing in a thin layer of 5% polyacrylamide gel (3% cross-linked) containing 2% carrier ampholytes (ampholine pH 3.5–10) was performed with an LKB 2117 Multiphor unit according to the directions of the manufacturer (LKB-Producter AB, Sweden). Gels were fixed overnight in 12% trichloroacetic acid then rinsed in several changes of water to remove carrier ampholytes before staining with 0.02% Coomassie Blue in methanol-acetic acid-water (45:9:46) and destaining with a solution of methanol (25%) and acetic acid (10%). Preparative

isoelectric focusing of 1 $D_{54-60}$ IM3El 2–3 (1.6 mg N) was done in a 110 ml LKB column at 4°C using 3% carrier ampholytes (pH 3.5–10) in a sucrose gradient as described by the manufacturer. Electrofocusing was for 3 days with a final potential of 700 V.

*Equilibrium Dialysis.* Equilibrium dialysis and displacement experiments were carried out in lucite microdialysis chambers (75) at 25°C ± 0.1° and PBS was the buffer in all experiments. Dialysis tubing was boiled in 2 M $Na_2CO_3$ and rinsed thoroughly before use. Control experiments (4) established that there was no detectable binding of the ligand [$^3$H]IM7-OH to the dialysis membrane, to normal human γ-globulin (4.1 mg/ml) or to the levan-reactive IgA mouse myeloma protein Y5476 (14 mg/ml) (55). Experiments were done with equal volumes (50 or 75 μl) on each side of the membrane and dialysis cells were allowed to reach equilibrium over a 72-h period of continuous mixing; however, displacement experiments with protein QUPC 52 were given an extra day because of the high concentrations of competitors employed. Control experiments indicated that equilibrium was probably attained in less than 24 h. Samples (20 μl) for radioactivity measurements were taken from the hapten side only (76) and counted in duplicate or triplicate. The [$^3$H]IM7-OH gave about 2.9 × $10^6$ cpm/μmol in Bray's solution (77) and 3.5 × $10^6$ cpm/μmol in Insta-Gel (Packard Instrument Co., Downers Grove, Ill.). In displacement experiments, nonlabeled competitors did not cause any detectable quenching of counts except for the highest amounts of methyl α D glucoside employed with protein QUPC 52 and this occurred only in Insta-Gel and was corrected for. The free concentration of competitor was calculated by the method of Nisonoff and Pressman (78).

*Fluorescence Titrations.* Fluorescence titrations were performed with square quartz semimicro cuvettes (5 mm light path, 0.6 ml capacity) and an Aminco-Bowman spectrophotofluorometer equipped with a cell jacket thermostated at 25°C (68). Haptens were added in 5–20 μl volumes to 250 μl portions of protein solutions having optical densities of about 0.18 (1 cm light path) at 280 nm. None of the haptens absorbed in the 280 nm region and the excitation wave length was set between 280 and 285 nm depending on the protein and emission was measured between 345 and 350 nm. Values for $Q_{max}$ were determined at the end of each titration by averaging the results from two additions of a concentrated ligand solution, each resulting in a greater than 97% saturation of sites. All titrations were done against a reference cell containing the solution being titrated and in this way it was possible to detect and correct for instrument drift. It was also necessary to plan experiments so that each addition of hapten resulted in at least a 3% quenching of fluorescence after correction for dilution.

## Results

*Isoelectric Focusing of Human Antibodies.* Purified antidextrans from 1 $D_{54-60}$ were 90–95% reactive with Sephadex G75 and analytical isoelectric focusing revealed a restriction in heterogeneity as compared with whole γ-globulin from the same individual. The patterns for antidextrans eluted with IM3 (Fig. 1 A,IM3El 1, IM3El 2–3, and IM3El 4–6) were similar with three major bands near pH 7.0 and several less prominent bands between pH 6.0 and pH 8.5. The antidextrans eluted with IM6 (Fig. 1 A, IM6El 1–6) were qualitatively like the IM3 eluates, but the three major bands near pH 7.0 were less obvious while those between pH 7.0 and pH 8.0 accounted for a greater proportion of the total protein. Purified antilevan from 1 $D_{54-60}$ (Fig. 1 A, L) and antibodies to blood group A substance from 1 $D_{33-34}$ (Fig. 1 A, A) also were restricted in heterogeneity and visibly different from each other and from the 1 $D_{54-60}$ antidextrans. The anti-A antibodies had relatively low isoelectric points. A pH 3.5 to pH 10 gradient was used for the preparative separation of 1 $D_{54-60}$ IM3El 2–3 into seven fractions (Fig. 1 B and C) which were studied later.

*Equilibrium Dialysis of Mouse Myeloma Proteins and Human Antidextrans.* Purified BALB/c mouse myeloma proteins W3129 and QUPC 52 were approximately 95% reactive with Sephadex G75, had two binding sites for [$^3$H]IM7-OH per IgA monomer, and gave linear Scatchard plots indicating homogeneous bind-

DOCKET ALARM

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

**LAW FIRMS**
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

**FINANCIAL INSTITUTIONS**
Litigation and bankruptcy checks for companies and debtors.

**E-DISCOVERY AND LEGAL VENDORS**
Sync your system to PACER to automate legal marketing.