IMMUNOCHEMICAL SPECIFICITY OF THE COMBINING SITE OF MURINE MYELOMA PROTEIN CAL20 TEPC1035 REACTIVE WITH DEXTRANS*

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Murine myeloma proteins with different specificities are produced by induction of plasmacytomas in BALB/c and NZB mice after intraperitoneal injection of mineral oil or solid plastic material (1–7). Their specificities have been characterized by their ability to react with various antigens such as dextran, levan, $\beta(1\rightarrow6)$-D-galactan, nucleic acid, pneumococcal C polysaccharide, serum lipoprotein, dinitrophenyl-substituted protein, glycoproteins with nonreducing N-acetyl-D-glucosamine (8) and proteins containing artificially introduced N-acetyl-D-glucosamine residues (8). The specificities and combining site sizes of BALB/c and NZB myeloma antidextrans have been extensively studied by quantitative precipitin inhibition assays (9–12) and have been confirmed by measurements of their association constants by equilibrium dialysis (13, 14), fluorescence quenching (13, 15), and affinity electrophoresis (16, 17). The cavity-type combining site of the BALB/c anti-dextran W3129 specific for terminal nonreducing chains of $\alpha(1\rightarrow6)$-linked dextrans is most complementary to isomaltopentaose (11, 13, 15, 16), whereas the groove-type combining sites of BALB/c QUPC52 antidextran specific for internal chains of $\alpha(1\rightarrow6)$-linked glucoses was most complementary to isomaltohexaose (11, 13). NZB antidextrans PC3658 and PC3936 are also specific for internal $\alpha(1\rightarrow6)$ chains, although their combining site sizes are somewhat uncertain (12). With BALB/c myeloma antidextrans specific for $\alpha(1\rightarrow3)$-linked glucose, nigerotriose showed the greatest complementarity with UPC102 and MOPC104E (9, 11), whereas nigeropentaose was best with J558 (10).

In the present investigation, the immunochemical specificity of the combining site of CAL20 TEPC1035, an IgG$_{2b}$ myeloma protein, reactive with dextrans having high proportions of $\alpha(1\rightarrow3)$ linkages has been studied and has also been compared with murine myeloma antidextrans reported previously. It has a combining site that differs from those of all other antidextrans studied.

Materials and Methods

Myeloma Proteins and Antiserum. CAL20 TEPC1035 was induced with Pristane (2,6,10,14-tetramethylpentadecane; 268 mol wt) by the method of Anderson and Potter (18) in a CAL20

mouse. CAL20 is an Ig BALB/c congenic strain that bears the AL/N allotype locus (19). CAL20 TEPC1035 was typed as an IgG2b,κ immunoglobulin by immunoelectrophoresis. The tumor was transplanted in (BALB/c × DBA/2)F₁ hybrid mice for production of ascites. Myeloma proteins J558 (IgA) MOPC104E (IgM) and UPC102 (IgA) have been described previously (9–11). Rabbit antiserum (serum 895) to DGlcα(1→6)DGlcα(1→4)DGlcα(1→4)DGLc was prepared by immunization with the oligosaccharide coupled to keyhole limpet hemocyanin (KLH)[1] via the phenethylamine-isothiocyanate derivative; the antigen used for testing was the oligosaccharide coupled to bovine serum albumin (BSA) (20, 21). Both antiserum and antigen were kindly provided by Dr. David A. Zopf of the National Institutes of Health, Bethesda, Md. (NIH). The oligosaccharide was coupled to BSA for use as antigen by reacting with β-(p-aminophenylethylamine) forming the N-alkylglycoside; this was reduced with NaBH₄ and converted to the isothiocyanate ,with thiophosgene and coupled to BSA. It contained four oligosaccharide units per molecule BSA. It will be referred to as panosyl-α(1→4)DGlcφNCS BSA. Isomaltose oligosaccharides coupled to BSA by bromine oxidation followed by the mixed anhydride reaction were (22) also available.

Dextrans. The dextrans used in the present study have been described previously (23–30). The proportions of α(1→6), α(1→3)-like, and α(1→4)-like linkages in the dextrans used are summarized in Table I according to the previous reports (24–26). The L-fractions from dextrans B1355, B1498, and B1501 were kindly provided by Dr. M. E. Slodki (Fermentation Laboratory, United States Department of Agriculture, Peoria, Ill.). Dextran B 742S was the preparation previously denoted as C3R.

Mono- and Oligosaccharides. Glucose, methyl α-D-glucoside, methyl-β-D-glucoside, trehalose, cellobiose, gentiobiose, kojibose, panose, isomaltose (IM2), isomaltotriose (IM3), isomaltopentaose (IM5), isomaltohexaose (IM6), isomaltoheptaose (IM7), nigerose (N2), nigerotriose (N3), nigerotetraose (N4), nigeropentaose (N5), nigerohexa-heptaose (N6,7), maltose (M2), maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and maltoheptaose (M7), and branched oligosaccharides whose structures are shown in Fig. 1 have been described previously (10, 11, 17, 27, 28). Isopanosylα(1→4)isopanose isolated from pullulan was kindly provided by Dr. T. Sawai, Aichi Kyoiku University and three trisaccharides: DGlcα(1→6)-DGlcα(1→3)DGlc, DGlcα(1→3)DGlcα(1→6)DGlc, andDDGlcα(1→2)DGlcα(1→6)DGlc (29) and a pentasaccharide DGlcα(1→2)DGlcα(1→6)DGlcα(1→6)DGlcα(1→6)DGlc from Dr. M. Torii, Osaka University, Osaka, Japan (31). A tetrasaccharide DGlcα(1→6)DGlcα(1→4)-DGlcα(1→4)DGlc was a gift of Dr. A. Lundblad, University of Lund, Sweden through Dr. David Zopf.

Immunochemical Assay. Quantitative precipitin and precipitin inhibition assays were performed by a microprecipitin technique (30) using a final vol of 400 µl; 6.5 µg of antibody N were mixed with varying amounts of antigen for quantitative precipitin assays; for quantitative precipitin inhibition assays, known quantities of sugars were added to 6.5 µg of antibody N and 40 µg dextran B1498S, an amount giving maximum precipitation in the absence of inhibitor; 15 µg dextran B1498S, 25 µg dextran B1498S and 15 µg dextran B1355S were used with J558, MOPC104E, and UPC102, respectively. The mixtures were incubated at 37°C for 1 h and then kept at 4°C for 1 wk. The total nitrogen content in the washed precipitate was determined by ninhydrin procedure (32).

Calculation of Association Constant and Free Energies. As reported previously (16, 17), the association constants were determined by affinity electrophoresis from the extent of retardation or restoration of the ligand-specific band as a function of the concentration of inhibitor in the gel. To determine the association constant (K^a) of dextran to myeloma protein CAL20 TEPC1035, dextran at an appropriate concentration was added to the separating gel. For determination of the association constant of inhibitor (K_i^a), varying concentrations of inhibitor were added to the gel together with an amount of dextran B1498S (final concentration 120 µg/ml) giving 50% retardation of the dextran-specific band and restoration of the mobility of the retarded dextran-specific band by inhibitor was measured. 3.7 µg ascitic fluid protein per tube was used for determination of K^a and K_i^a. The free energy of the oligosaccharide-myeloma protein interaction was calculated according to the previous reports (13, 33).

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; K^a, association constant(s); K_i^a, association constant(s) of inhibition; KLH, keyhole limpet hemocyanin; NMR, nuclear magnetic resonance.
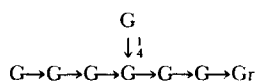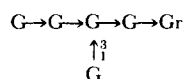
4^3-α-D-glucosylisomaltopentaose (II/3):
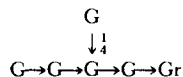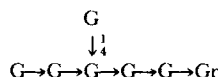
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            G
            ↓⁴₁
G→G→G→G→Gr
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4^4-α-D-glucosylisomaltohexaose (II/4):

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               G
               ↓⁴₁
G→G→G→G→G→Gr
```

4^4-α-D-glucosylisomaltoheptaose (II/5):

```
               G
               ↓⁴₁
G→G→G→G→G→G→Gr
```

3^3-α-D-glucosylisomaltopentaose (I/4):

```
G→G→G→G→Gr
      ↑³₁
      G
```

3^4-α-D-glucosylisomaltohexaose (I/5):

```
G→G→G→G→G→Gr
         ↑³₁
         G
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3^4-α-D-glucosylisomaltoheptaose (I/6):

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G→G→G→G→G→G→Gr
            ↑³₁
            G
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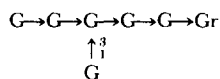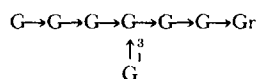
Fig. 1. Structures of branched oligosaccharides. G, D-glucopyranosyl; Gr, reducing D-glucose; →, α-D-(1→6) link; ↑, α-D-(1→3) link; ↓, α-D-(1→4) link. Superscripts denote the substituted glucose residue, counting from the reducing end.

Results

Quantitative Precipitin Assay. Fig. 2 shows the quantitative precipitin curves of myeloma proteins CAL20 TEPC1035 and J558 and serum 895 with various dextrans and the results are summarized in Table I. As shown in Fig. 2 A, only three dextrans, B1498S, B1355S4, and B1501S, with high proportions of α(1→3) linkages precipitated myeloma protein CAL20 TEPC1035. None of 15 other dextrans tested gave any precipitate. As described previously (10), the most potent precipitinogens for myeloma protein J558 were the same three dextrans B1498S, B1355S4, and B1501S (Table I). However, with J558, other dextrans reacted but to a lesser extent (10). The reactivity of dextrans B742S (C3R), B1375, and B1255 with myeloma protein J558 was two to three times less than those of the three most strongly reacting dextrans, whereas

TABLE I

Precipitating Power of Various Dextrans with Myeloma Proteins CAL20 TEPC1035, J558, and Serum 895

Symbol	Dextran	Linkages			CAL20 TEPC1035	K^a	J558	895
		1→6	1→3-like	1→4-like				
		%				ml/g		
⊞	B1355S4	57	35	8	+++*	4.42×10^3	+++	+++
⊟	B1142	67	29	4	–		–	+++
▲	B1498S	62	27	11	+++	5.02×10^3	+++	+++
⊡	B742S (C3R)	57	26	17	–		++	
▽	B1501S	67	20	13	+++	3.8×10^3	+++	+++
◕	B1141	79	18	3	–		–	
⊡	B1425	74	18	8	–		–	
△	B1375	81	13	6	–		++	
⊠	B1351	85	11	4	–		–	
◨	N279	96	4	0	–		–	+++
▼	B1498L	94	6	0	–		–	
▼	B1501L	93	7	0	–		–	
⊟	B1355L	95	2	3	–		–	
⋈	B1399	89	0	13	–		–	+++
○	B1255	86	0	14	–		++	+++
⊗	B742L	87	0	13	–		+	+++
●	B1254S[L̵]	77	0	23	–		+	
⊠	B1299S3	65	0	35	–		++	+++
○	Pullulan				–		–	++
⊙	Glycogen				–		–	++
◆	DGlcα(1→6)DGlcα(1→4)DGlcα(1→4)DGlc-φNCS-BSA				–		–	+++
■	IM3φNCS-BSA				–			+++
◆	IM6φNCS-BSA				–			+++
	IM2-CONH-BSA				–			–
	IM6-CONH-BSA				–			–

*+++, 6–7 µg N precipitated; ++, 2–3 µg N precipitated; +, 0.5–1 µg N precipitated; – no N precipitated.

dextran B1254S [L̵] and B742L were less active (Fig. 2 B and Table I). Both myeloma proteins CAL20 TEPC1035 and J558 gave no precipitation with yeast pullulan and rabbit liver glycogen (Table I), nor did they precipitate with panosyl-α(1→4)DGlcNCS-BSA, IM3-φNCS-BSA, IM6-φNCS-BSA, IM2-CONH-BSA, and IM6-CONH-BSA in precipitin assays or in gel diffusion tests, although anti-panosyl-α(1→4)DGlc (serum 895) precipitated well with panosyl-α(1→4)DGlc-φNCS-BSA, IM6-φNCS-BSA, and all dextrans used as shown in Fig. 2C. Glycogen, pullulan, and IM3-φNCS-BSA precipitated poorly with anti-panosyl-α(1→4)DGlc.

Quantitative Precipitin Inhibition Assay. Fig. 3 and Table II present the ability of various sugars to inhibit the precipitation reaction between myeloma protein CAL20 TEPC1035 and dextran B1498S. Among various sugars tested, the best inhibitor was panose DGlcα(1→6)DGlcα(1→4)DGlc which was 8, 23, and 42 times more active than maltose, nigerose, and isomaltose, respectively. A tetrasaccharide DGlcα(1→6)-DGlcα(1→4)DGlcα(1→4)DGlc was 1.5 times more potent than a trisaccharide DGlcα(1→6)DGlcα(1→3)DGlc but 3.3 times less active than panose. Of the trisaccharides with two kinds of linkage, those with terminal nonreducing α(1→6)-linked
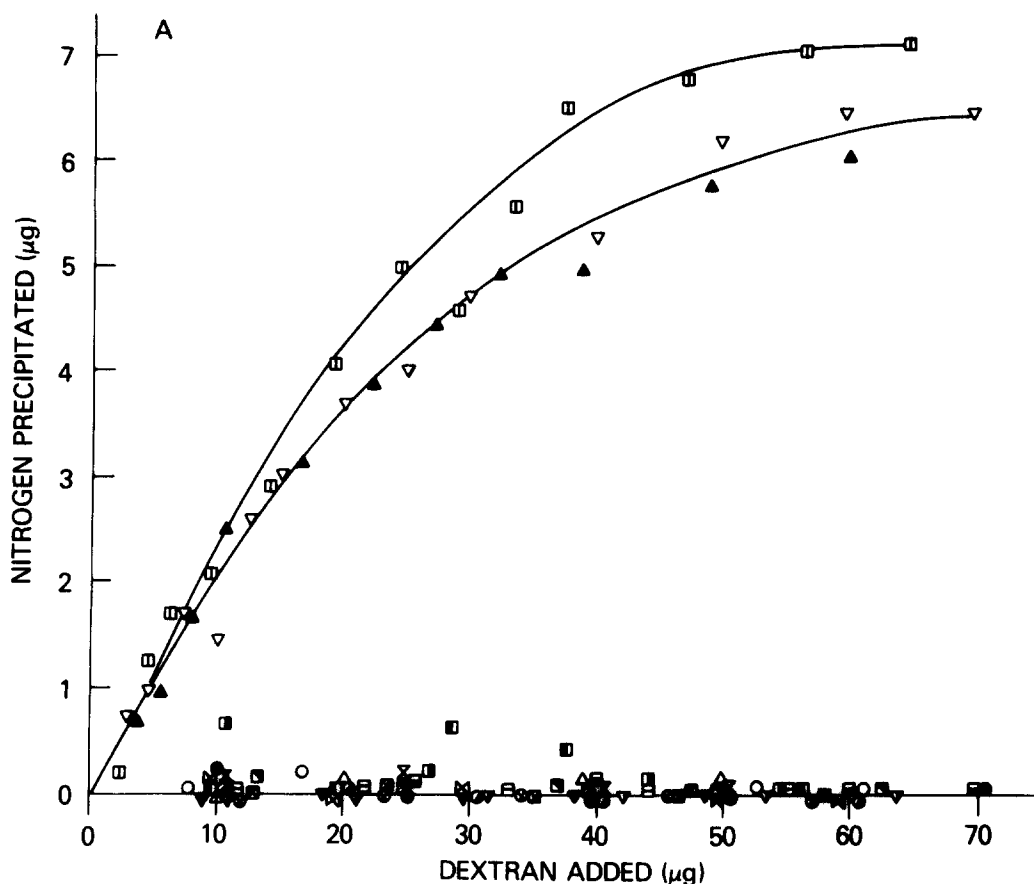
FIG. 2A

FIG. 2.   Quantitative precipitin curves of CAL20 TEPC1035 (A), J558 (B), and antiserum 895 (C) with various dextrans and antigens. 50 μl of CAL20 TEPC1035 (1:20), 50 μl of J558 (1:2), and 80 μl of serum 895 (undiluted) were used. Total vol in all cases was 400 μl. Symbols are shown in Table I. B1498S and B1501S were previously found to react comparably to B1355S (10).

glucose were more active than those with terminal nonreducing glucose linked α-(1→3), α(1→4), or α(1→2). Isopanosylα(1→4)isopanose, DGlcα(1→4)DGlcα(1→6)-DGlcα(1→4)DGlcα(1→4)DGlcα(1→6)DGlc, was 2.85 times more potent than the iso-panose, DGlcα(1→4)DGlcα(1→6)DGlc; the latter being as active as methyl α-D-glucoside. With α(1→4)-linked glucosyloligosaccharides from maltose (M2) to mal-toheptaose (M7), the order of inhibitory potency was: maltoheptaose (M7) = mal-tohexaose (M6) > maltopentaose (M5) > maltotetraose (M4) = maltotriose (M3) = maltose (M2). M7 and M6 were as potent as the tetrasaccharide DGlcα(1→6)DGlcα-(1→4)DGlcα(1→4)DGlc. The α(1→3)-linked glucosyloligosaccharides from nigerose (N2) to nigerohexa-heptaose (N6,N7) were about the same in inhibitory potency, but were about one-third as active as α(1→4)-linked glucosyloligosaccharides. Branched oligosaccharides were as active or a little less active than maltose, but more potent than nigerose. Isomaltosyloligosaccharides were much less potent inhibitors and isomaltopentaose (IM5), isomaltohexaose (IM6) and isomaltoheptaose (IM7) showed
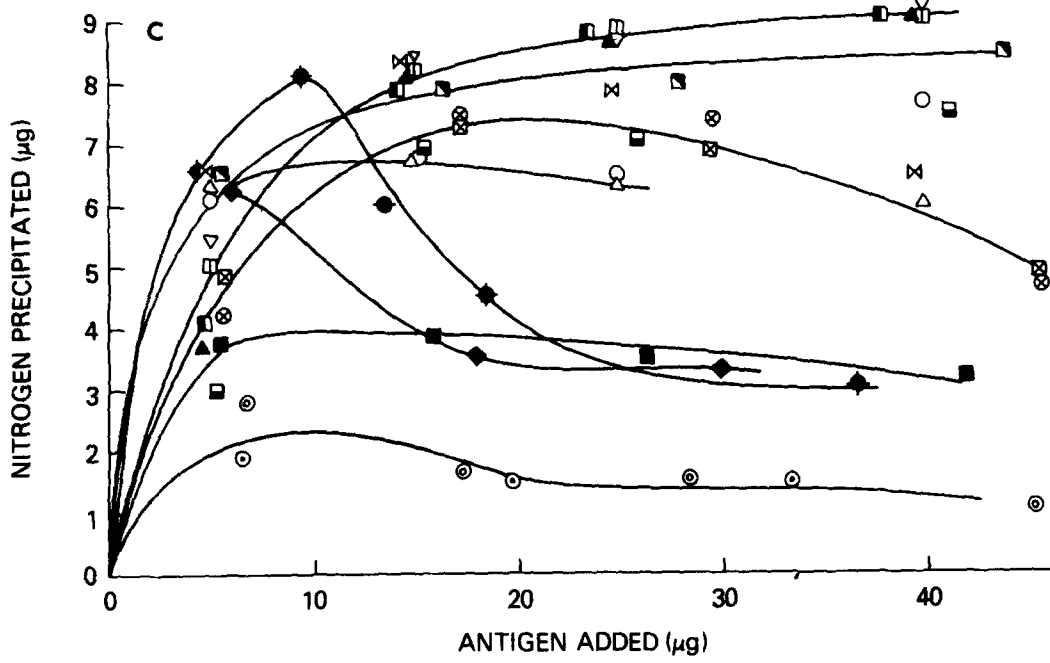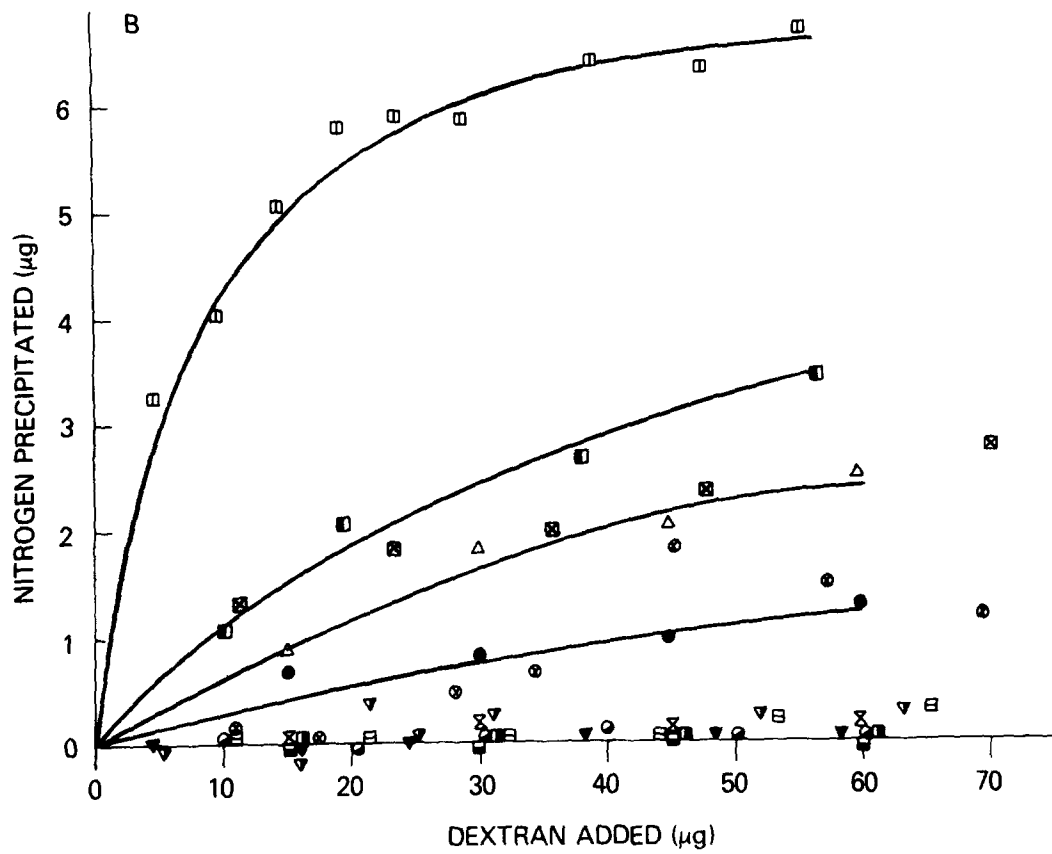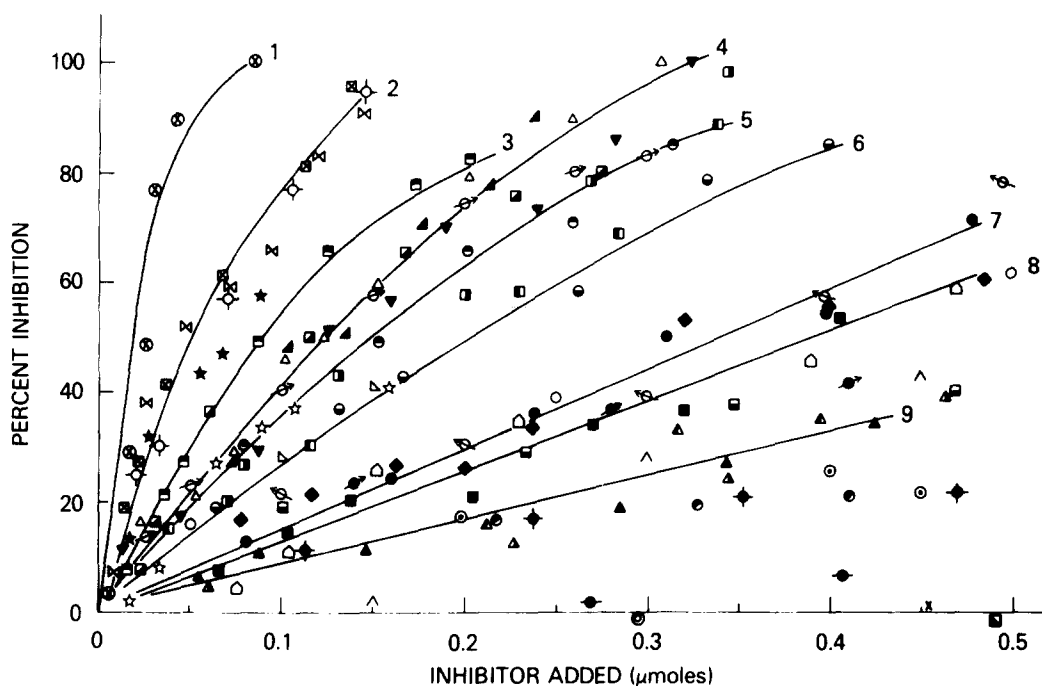
Fig. 2 B and C

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Fig. 3. Inhibition by various oligosaccharides of precipitation of 50 μl of CAL20 TEPC1035 (1:
20) with dextran B1498S (40 μg). Total vol was 400 μl. Symbols are shown in Table II.

44% inhibition at 0.5 μmol, 20% inhibition at 0.41 μmol and 35% inhibition at 0.4
μmol, respectively. An unusual finding was that isomaltose was only one-half, nigerose
was equally, and maltose was three times as active as methyl α-D-glucoside. Maltose,
methyl α-D-glucoside, and isomaltose were more active than their corresponding β-
linked anomers such as cellobiose, methyl β-D-glucoside, and gentiobiose. 6-O-acetyl-
glucose was more potent than glucose. Glucose, kojibiose, and methyl β-D-glucoside
gave 22% inhibition at 0.2 μmol, 20% inhibition at 0.98 μmol, and 30% inhibition at
1.1 μmol, respectively. Trehalose showed no inhibition up to 1.1 μmol. A trisaccha-
ride DGlcα(1→2)DGlcα(1→6)DGlc and a pentasaccharide DGlcα(1→2)DGlcα-
(1→6)DGlcα(1→6)DGlcα(1→6)DGlc showed only 15% inhibition at 0.36 μmol and
0.29 μmol, respectively. With myeloma proteins J558, UPC102, and MOPC104E,
nigerose was a better inhibitor than panose and maltose. Nigerose with J558, UPC102,
and MOPC104E showed 42% inhibition at 0.8 μmol, 55% inhibition at 0.45 μmol and
25% inhibition at 0.9 μmol, whereas panose gave 15% inhibition at 0.99 μmol, 15%
inhibition at 0.52 μmol, and no inhibition at up to 0.99 μmol, respectively. With
MOPC104E, nigerotetraose gave 75% inhibition at 0.82 μmol.

Association Constants.   In affinity electrophoresis, the mobility of one dextran-specific
band in myeloma ascitic fluid CAL20 TEPC1035 was retarded proportionally to
dextran concentration when dextran was added to the gel as illustrated previously
(16, 17). Among different dextrans used, only three dextrans which precipitated,
B1498S, B1355S4, and B1501S retarded the mobility of the dextran-specific band in
CAL20 TEPC1035 myeloma ascitic fluid; other dextrans, N279, B742S (C3R), B742L,
B1425, B1255, B1299S3, B1375, and B1254S[Ł], did not show any retardation up to

TABLE II

Inhibitory Potency of Oligosaccharides of Precipitation of CAL20 TEPC1035 by Dextran B1498S

Symbol	Inhibitor	Curve No.	μmol giving 50% inhibition	Relative potency
⊗	Panose DGlcα(1→6)DGlcα(1→4)DGlc	1	0.015	8.0
◇	DGlcα(1→6)DGlcα(1→4)DGlcα(1→4)-DGlc	2	0.05	2.4
⊠	Maltoheptaose (M7)	2	0.05	2.4
⋈	Maltohexaose (M6)	2	0.05	2.4
★	DGlcα(1→6)DGlcα(1→3)DGlc		0.075	1.6
▬	Maltopentaose (M5)	3	0.085	1.41
∅	Isopanosylα(1→4)isopanose, DGlcα-(1→4)DGlcα(1→6)DGlcα(1→4)-DGlcα(1→4)DGlcα(1→6)DGlc	4	0.12	1.0
◪	Maltotetraose (M4)	4	0.12	1.0
△	Maltotriose (M3)	4	0.12	1.0
▼	Maltose (M2)	4	0.12	1.0
◢	4^4-α-D-glucosylisomaltohexaose (II/4)	4	0.12	1.0
◻	4^4-α-D-glucosylisomaltoheptaose (II/5)	5	0.15	0.8
⬤	4^3-α-D-glucosylisomaltopentaose (II/3)	5	0.15	0.8
◺	3^4-α-D-glucosylisomaltohexaose (I/5)		>0.15 (47% inhibition)	—
☆	DGlcα(1→3)DGlcα(1→6)DGlc		>0.16 (40% inhibition)	—
◖	3^3-α-D-glucosylisomaltopentaose (I/4)	6	0.205	0.59
◧	3^4-α-D-glucosylisomaltoheptaose (I/6)	6	0.205	0.59
◆	Nigerose (N2)	7	0.34	0.35
●	Methyl α-D-glucoside	7	0.34	0.35
◳	Isopanose (DGlcα(1→4)DGlcα(1→6)DGlc	7	0.34	0.35
○	Nigerohexa-heptaose (N6,N7)	7	0.36	0.33
⌂	Nigeropentaose (N5)	8	0.42	0.29
■	Nigerotetraose (N4)	8	0.42	0.29
▲	Nigerotriose (N3)	8	0.42	0.29
⬗	Cellobiose DGlcβ(1→4)DGlc	8	0.5	0.24
▲	Isomaltotriose (IM3)	9	0.6	0.20
∧	Isomaltose (IM2)	9	0.64	0.19
▲	Isomaltoheptaose (IM7)		>0.4 (35% inhibition)	—
◕	Isomaltohexaose (IM6)		>0.41 (20% inhibition)	—
▤	Isomaltopentaose (IM5)		>0.59 (44% inhibition)	—
⊙	6-O-acetyl-glucose		0.84	0.14
◆	Gentiobiose DGlcβ(1→6)DGlc		0.95	0.12
✕	Glucose		22% inhibition at 0.92 μmol	
◎	Kojibiose DGlcα(1→2)DGlc		20% inhibition at 0.98 μmol	
◆	Methyl β-D-glucoside		30% inhibition at 1.1 μmol	
◣	Trehalose DGlcα(1→1)DGlc		No inhibition at 1.1 μmol	
	DGlcα(1→2)DGlcα(1→6)DGlc		15% inhibition at 0.36 μmol	
	DGlcα(1→2)DGlcα(1→6)-DGlcα(1→6)DGlcα(1→6)DGlc		14% inhibition at 0.29 μmol	

500 μg/ml. The addition of these dextrans at >500 μg/ml retarded the dextran-specific band but also retarded the albumin band in the ascitic fluid, indicating that retardation was nonspecific. The K^a of myeloma protein CAL20 TEPC1035 was 5.02 × 10^3 ml/g for B1498S, 4.42 × 10^3 ml/g for B1355S4, and 3.8 × 10^3 ml/g for B1501S,

TABLE III

$K_i{}^a$ (M^{-1}) of Glucosyloligosaccharides to CAL20 TEPC1035 Determined by
Affinity Electrophoresis

Oligosaccharide	$K_i{}^a$	ΔF_0 (Kcal)	Percentage
Panose	8.19×10^3	−5.32	100
Maltotriose	1.54×10^3	−4.34	81
Maltose	2.34×10^3	−4.58	86
Methyl-α-D-glucoside	7.2×10^2	−3.89	73

respectively. On addition to the gel of dextran B1498S together with an inhibitor, the retarded dextran-specific band in myeloma ascitic fluid CAL20 TEPC1035 was restored proportionally to the inhibitor concentration as illustrated previously (16). The $K_i{}^a$ of oligosaccharide inhibitors were: 8.19×10^3 M^{-1} for panose, 1.54×10^3 M^{-1} for maltotriose, 2.34×10^3 M^{-1} for maltose, and 7.2×10^2 M^{-1} for methyl α-D-glucoside, respectively.

The binding energy of oligosaccharides was calculated from the $K_i{}^a$ determined by affinity electrophoresis and are shown in Table III; maltose and maltotriose had 81 and 86%; and methyl α-D-glucoside had 73% of the total binding energy of panose.

Discussion

Previously described BALB/c and NZB myeloma antidextrans have shown specificity for either α(1→6)-linked or α(1→3)-linked oligosaccharides of glucose (9–12). The present findings demonstrate that myeloma protein CAL20 TEPC1035 is an antidextran of a different specificity from any previously described antidextran.

Only three dextrans, B1498S, B1355S4, and B1501S, with high proportions of α(1→3) linkages as shown in Table I precipitate myeloma protein CAL20 TEPC1035. These results might suggest that myeloma protein CAL20 TEPC1035 might be strictly α(1→3) specific. However inhibition studies create problems with such an interpretation.

The best inhibitor was panose, DGlcα(1→6)DGlcα(1→4)DGlc which was 2.4, 6.9, and 12.6 times more potent than maltose, nigerose, and isomaltose, respectively. As shown in Table II and Fig. 3, α(1→3)-linked glucosyloligosaccharides were much less active than panose and α(1→4)-linked glucosyloligosaccharides.

As previously reported (9–11) the three dextrans B1498S, B1355S4, and B1501S were also the most potent precipitinogens for myeloma antidextrans J558, UPC102, and MOPC104E, although dextrans B742S (C3R), B1255, B1299S, B1254S[Ł] and B1398 also precipitate myeloma proteins J558, UPC102, and MOPC104E but to a lesser extent (9–11).

However the best inhibitors for myeloma proteins J558, UPC102, and MOPC104E were α(1→3)-linked glucosyloligosaccharides, whereas panose and maltose were less active. The relative potency of panose at 50% inhibitor relative to nigerose as 1.0 on a molar basis was 0.105 with MOPC104E (9); with UPC102, 0.52 µmol panose gave only 15% inhibition as compared to 56% with 0.45 µmol nigerose. With J558, 0.99 µmol panose gave 15% inhibition whereas 0.8 µmol nigerose gave 41% inhibition. Thus the specificity of the combining site of myeloma protein CAL20 TEPC1035 is clearly different from those of myeloma proteins J558, UPC102, and MOPC104E.

Panose, DGlcα(1→6)DGlcα(1→4)DGlc, was 2.5 times more potent than a tetrasac-

charide DGlcα(1→6)DGlcα(1→4)DGlcα(1→4)DGlc, indicating that the combining site size of myeloma protein CAL20 TEPC1035 is at least as large as a trisaccharide, and that a third linkage when α(1→4) reduces access to the combining site. With the oligosaccharides available it is not possible to decide whether inhibiting power would increase if the panosyl moiety were linked other than α(1→4) to an additional glucose. Thus the data only establish a minimum estimate for the combining site size. Because one seldom, if ever, can be certain with myeloma proteins of the specificity of the antigens that induce the clone, a precise definition of the complementarity of the site is only obtainable when all possible related oligosaccharides have been tested.

The combining site of myeloma protein CAL20 TEPC1035 is specific for α-linked glucose because maltose, isomaltose, and methyl α-D-glucoside were more potent inhibitors than the corresponding β-linked anomers, cellobiose, gentiobiose, and methyl β-D-glucoside. Among α-linked trisaccharides with two kinds of linkage tested, those with a terminal nonreducing α(1→6) linkage were more inhibitory than those with either α(1→3), α(1→4), or α(1→2) linkages because panose and the trisaccharide DGlcα(1→6)DGlcα(1→3)DGlc were more specific for myeloma protein CAL20 TEPC1035 than the corresponding isomers, isopanose and DGlcα(1→3)DGlcα(1→6)-DGlc, respectively. The linkage of the second to the third sugar is thus also important for binding and the order of inhibitor potency was: 1→4 > 1→3 > 1→6 as shown in Table II. These findings clearly show that the conformation of the combining site is highly specific. Inhibition tests with α(1→4)-linked glucosyl oligosaccharides showed the order of inhibitory potency to be: maltoheptaose (M7) = maltohexaose (M6) > maltopentaose (M5) > maltotetraose (M4) = maltotriose (M3) = maltose (M2). A similar order of inhibition was obtained with isomaltose oligosaccharides in inhibition tests with BALB/c and NZB myeloma antidextrans with groove-type sites of α(1→6) specificity (11, 12). The increased potency of M7 and M6 as compared with M2 and M3 may be attributable to the longer sugar chains having an effective partial bivalence e.g., to their ability to interact with the site in several ways. The failure of CAL20 TEPC1035 to precipitate with glycogen is surprising. However, the average length of the α(1→4) chains in glycogen per α(1→6) branch point is ~14 units (34) so that these would show a considerable tendency to form helices, which would not provide an optimal conformation for precipitation. Indeed, in inhibition studies with human antidextran, 9D4, M3, M4, and M5 were equally active on a molar basis, whereas M7, the smallest oligosaccharide theoretically capable of forming one turn of a helix was somewhat less active (35).

In addition to these findings, the isopanosylα(1→4) isopanose, DGlcα(1→4)DGlcα-(1→6)DGlcα(1→4)DGlcα(1→4)DGlcα(1→6)DGlc was more potent than isopanose DGlcα(1→4)DGlcα(1→6)DGlc. If the site was entirely groove-type, one might have expected the isopanosyl α(1→4) isopanose to react as well as panose, the nonreducing terminal DGlcα(1→4) being outside with the internal →4DGlcα(1→6)DGlcα(1→4)-DGlcα- moiety occupying the same position as panose in the site. As this was not the case and as the isopanosyl α(1→4) isopanose was only as active as maltose, M3, and M4, the higher activity of the isopanosyl α(1→4) isopanose as compared with isopanose is ascribable to interaction with the internal maltosyl moieties. Panose would react optimally in the site, whereas M2 to M5 and the isopanosyl α(1→4) isopanose would interact only with the groove subsite. The K_i^a of methyl α-D-glucoside was 7.2 × 10² for CAL20 TEPC1035 comparable to that of 9.4 × 10² for myeloma

protein W3129 which has a cavity type site complementary to a pentasaccharide. Further, this K_i^a is 600 times higher than that, 1.6×10^0, for QUPC52 with a groove-type site complementary to a hexasaccharide (13). The K_i^a of methyl α-D-glucoside for CAL20 TEPC1035 was 73% that of panose; in W3129 it was 1.5%; and in QUPC52 it was 0.23% that of IM3. Thus, the terminal nonreducing glucose linked α contributes predominantly to binding with CAL20 TEPC1035. These findings suggest that the combining site of myeloma antidextran CAL20 TEPC1035 may be a partial cavity-type with the terminal nonreducing DGlc being held in three dimensions with the next two sugars in a groove.

However, relative to methyl α-D-glucoside, isomaltose was only one-half as potent as an inhibitor, whereas nigerose was equally active, and maltose was about three times as active. The difference between methyl α-D-glucoside and isomaltose is small and could be a consequence of isomaltose assuming an unfavorable conformation alone whereas in nigerose, maltose, and panose it would exist in a more restricted conformation.

As shown above, myeloma protein CAL20 TEPC1035 only precipitated three dextrans with high proportions of $\alpha(1\rightarrow3)$ linkages although its combining site was not most complementary to $\alpha(1\rightarrow3)$-linked glucoses and although $\alpha(1\rightarrow4)$-linked glucosyloligosaccharides show more affinity for the combining site than $\alpha(1\rightarrow3)$-linked glucosyloligosaccharides. Dextrans B1254S[Ł] and B742L did not precipitate myeloma protein CAL20 TEPC1035.
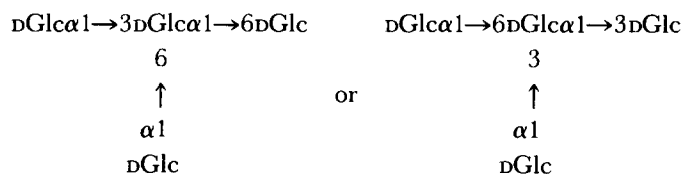
Recent structural studies (25, 36–40) propose, on the basis of nuclear magnetic resonance (NMR) data, that dextrans B1498S, B1355S4, and B1501S are comb-like structures made up of a linear backbone of [-3DGlcα(1$\rightarrow$6)DGlcα(1$\rightarrow$3)DGlcl-]$_n$ with short side chains of one or more glucoses linked $\alpha(1\rightarrow6)$ to the 3-linked glucoses of the main chain occurring about one every four or six [-3DGlcα(1$\rightarrow$6)DGlcα(1$\rightarrow$3)DGlcl-] units. If the site is a partial cavity as hypothesized, the high reactivity of these dextrans might be ascribed to the unsubstituted [-6DGlcα(1$\rightarrow$3)αDGlc-] portion of the backbone repeat reacting in the groove portion of the site complementary to the subterminal DGlc. However, it is also possible that the nonreducing terminal DGlcα(1$\rightarrow$6) unit of the side chains of the three dextrans might be reacting in the cavity-portion of the site. In both instances precipitation would be a result of multivalence. All of the branched oligosaccharides studied had DGlcα(1$\rightarrow$4) or DGlcα(1$\rightarrow$3) side chains linked to an $\alpha(1\rightarrow6)$ backbone and as inhibitors those with DGlcα(1$\rightarrow$4) branches were somewhat better than those with DGlcα(1$\rightarrow$3) branches.

Thus it is quite surprising that CAL20 TEPC1035 did not precipitate with dextrans B1254S[Ł] and B742L which contain DGlc branches linked $\alpha(1\rightarrow4)$ to long chains of a DGlcα(1$\rightarrow$6)-linked backbone (38), thus resembling the 4-linked isomaltosyl oligosaccharides which inhibited quite well. Antiserum 895 (anti-panosylα-(1$\rightarrow$4)DGlcϕNCS-BSA) reacted well with its homologous antigen and with dextrans having different proportions of various linkages whereas CAL20 TEPC1035 was precipitated only by dextrans B1355S4, B1498S, and B1501S although its combining site shows a high specificity for the tetrasaccharide panosyl-$\alpha(1\rightarrow4)$DGlc. The nonreactivity of these dextrans may be a result of their molecular configuration blocking access to the small combining site of CAL20 TEPC1035. This is consistent with the findings that the isopanosylα(1$\rightarrow$4)isopanose obtained from pullulan was a good inhibitor but pullulan itself was not a precipitinogen for CAL20 TEPC1035. On the

other hand the weak cross reactivity of B1254S[L] and B742L with the $\alpha(1\rightarrow3)$-specific anti-dextrans J558, UPC102, and MOPC104E would be accounted for if the $\alpha(1\rightarrow4)$ branches could be accommodated in an $\alpha(1\rightarrow3)$-specific site. This was indeed found to be the case with anti-B1355S4 LAT_2 (10); this antiserum after absorption with B512 was inhibited by 4^4 α-D-glucosylisomaltohexaose and 4^3 α-D-glucosyliso-maltotetraose only slightly less well than by 3^4 α-D-glucosylisomaltohexaose and 3^3 α-D-glucosylisomaltotetraose. The failure of the panosyl-$\alpha(1\rightarrow4)$DGlcϕNCS-BSA to precipitate with CAL20 TEPC1035 is surprising. The antigen has only four glycosyl groups per molecule. It is possible that these are not distributed in a manner allowing precipitation, or that more groups are necessary for precipitation, or that if interaction occurs the complexes are soluble or result from steric effects.

A partial cavity type site complementary to at least a terminal nonreducing trisaccharide unit DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow4)$DGlc- would appear to account satisfactorily for all of the inhibition data (Fig. 3). Thus panose would be best with the terminal nonreducing DGlc$\alpha(1\rightarrow6)$ moiety held in three dimensions in the cavity subsite and contributing most of the binding energy (73% that of panose itself) with the rest of the site being a groove accessible to internal maltosyl linkages (13). The failure of the other dextrans to cross-react would be a consequence of their very different structures, whereas the reactivity of CAL20 TEPC1035 with the three dextrans with long chains of DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow3)$ would be accountable for as either of the two alternatives presented above. It would also be consistent with the increased inhibitory potency of M6 and M7 over M2 and M3 being ascribable to bivalence of the oligosaccharide. If the NMR data on the three dextrans that precipitate with CAL20 TEPC1035 do not unequivocally exclude the presence of some DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow3)$ side chains on the comb-like backbone,[2] this would provide an additional much better alternative because DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow3)$DGlc is a very good inhibitor and would appear to be reacting with the entire site but not fitting as well as panose. Indeed with B1355S from methylation, enzymatic and immunochemical studies Torii et al. (31) have proposed an alternative tentative structure with chains having terminal nonreducing DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow3)$DGlc units which would account for one of two specificities in rabbit antisera to B1355S as well as be consistent with the present interpretation for the reactivity of CAL20 TEPC1035.

It must be emphasized that the above interpretations are made on the basis of the limited data available and assume that no oligosaccharides with $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow3)$ linkages more potent than panose will be found. Alternative possibilities might be that the site is directed toward structures such as

$$\begin{array}{ccc}
\text{DGlc}\alpha1\rightarrow3\text{DGlc}\alpha1\rightarrow6\text{DGlc} & & \text{DGlc}\alpha1\rightarrow6\text{DGlc}\alpha1\rightarrow3\text{DGlc} \\
6 & & 3 \\
\uparrow & \text{or} & \uparrow \\
\alpha1 & & \alpha1 \\
\text{DGlc} & & \text{DGlc}
\end{array}$$

in which the essential requirement would be that the DGlc side chain be attached to

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[2] Note added in proof: Misaki et al. have recently proposed a structure consistent with the occurence of such side chains (Misaki, A., M. Torii, T. Sawai, and I. J. Goldstein. 1980. Structure of the dextran of *Leuconostoc mesenteroides* B1355. *Carbohydr. Res.* **84**:273.).

a disubstituted DGlc as was proposed (39) to account for higher activity of nigerotriose over nigerose with J558. If one of these compounds proves to be more active than panose this would be consistent with the K_i^a data on methyl α-D-glucoside and would account for the failure of IM3ϕCNS-BSA to precipitate. It would be highly desirable to study tetrasaccharides with these structures.

Another possibility might be that the site is entirely specific for the backbone requiring a tetra- penta- or hexasaccharide with alternating $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow3)$ linkages; this would make the site entirely groove-type and would not be consistent with the data on the high K_i^a of methyl α-D-glucoside as well as the binding data for the cavity type site of W3129 (13, 15) relative to the groove type of QUPC52. It should be noted, however, that higher K^a were found with antistreptococcal antibodies directed against internal sequences, e.g., groove-type sites of rhamnose with $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ linkages. Although this is attributable to the hydrophobic interaction with the CH_3 group of C6 of rhamnose (41, 42), enough data are not as yet available to correlate the magnitudes of binding constants precisely with site structure.

Should none of these alternatives prove to be correct and should panose remain as the best inhibitor, the possibility of the existence of another class of dextrans or polysaccharides with terminal nonreducing panosyl moieties than those currently known will have to be considered. It would also be of interest to study the antibody response of the CD mouse to one or more of the three dextrans with which CAL20 TEPC1035 reacts to determine whether induced anti-$\alpha(1\rightarrow3)$ dextran sites behave like that of CAL20 TEPC1035.

It should be remembered that heteroclitic antibody formation (e.g., an antibody which reacts more strongly to a related antigen than to the antigen used for immunization) in the mouse has been described (43); in this instance the C57BL/6 mouse immunized with a protein containing 4-hydroxy-3-nitrophenyl acetyl determinants produced antibody that reacted more strongly with derivatives in which an iodo- or a nitro-group was substituted on carbon 5 of the benzene ring than with the immunizing antigen; this heteroclitic response was under genetic control. It is conceivable that studies with B1355S might demonstrate a heteroclitic response.

The unusual nature of the combining site of CAL20 TEPC1035 make it desirable to sequence the molecule and to attempt to obtain crystals of an Fab fragment suitable for high-resolution x-ray crystallography.

Summary

The immunochemical specificity of the combining sites of murine myeloma protein CAL20 TEPC1035 was studied by quantitative precipitin and precipitin inhibition assays. Myeloma protein CAL20 TEPC1035 precipitated with only three dextrans, B1355S4, B1498S, and B1501S, with high proportions of $\alpha(1\rightarrow3)$ linkages, but not with any other dextrans, glycogen, and pullulan. Inhibition tests with various sugars show that the combining site of myeloma protein CAL20 TEPC1035 is most complementary to panose, a trisaccharide DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow4)$DGlc. Panose was 3.3 times more potent than a tetrasaccharide DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow4)$DGlc$\alpha(1\rightarrow4)$DGlc and 8, 23, 42, >42 times more active than maltose, nigerose, isomaltose, and kojibiose, respectively. These findings were paralleled by their binding properties as determined by affinity electrophoresis. The association constants (K^a) of these three dextrans to myeloma protein CAL20 TEPC1035 ranged from 3.8×10^3 ml/g to 5.02×10^3 ml/g.

The association constant of inhibitor (K_i^a) of panose was 8.19×10^3 M^{-1}. Myeloma protein CAL20 TEPC1035 is an antidextran with specificity different from those of other murine myeloma antidextrans and from human antidextrans reported previously, and its combining site size is at least as large as a trisaccharide. The binding constant of methyl α-D-glucoside (7.2×10^2) was 73% of that of panose and comparable to that of myeloma protein W3129 (9.4×10^2) with a cavity-type site and 600 times lower (1.6×10^0) for QUPC52 with a groove type site, indicating that the terminal nonreducing residue is held in a cavity. Inhibition data with various $\alpha(1\rightarrow4)$-linked oligosaccharides also indicate that the internal portions of these inhibitors may react directly with a portion of the combining site. These findings suggest that myeloma antidextran CAL20 TEPC1035 has a partial cavity-type combining site in which the terminal nonreducing DGlc$\alpha(1\rightarrow6)$ moiety is held in a cavity with the other two sugars forming a groove. However, oligosaccharides with one or more alternating [$\rightarrow$3DGlc$\alpha(1\rightarrow6)$DGlc$\alpha(1\rightarrow3)$DGlc$\rightarrow$] units with and without terminal nonreducing DGlc$\alpha(1\rightarrow6)$ or DGlc$\alpha(1\rightarrow3)$ side chains remain to be tested to determine whether structures known to be present in the three dextrans which precipitate CAL20 TEPC1035 may not prove to be more active than panose.

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