

IMMUNOCHEMICAL ANALYSIS OF THE IDIOTYPES OF MOUSE  
MYELOMA PROTEINS WITH SPECIFICITY FOR LEVAN  
OR DEXTRAN\*

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Brient and Nisonoff (1) showed that the interaction between certain antisera and variable region determinants of a given antibody is inhibited by ligands with which that antibody reacts specifically. These ligand-modifiable determinants constitute part of the total number of variable region determinants that have been called idiotypic determinants (2) or individual antigenic specificities (3). In the present investigation, the effect of ligand binding on this type of determinant (referred to as idio type) has been analyzed for a mouse myeloma protein with specificity for  $\alpha(1 \rightarrow 6)$  linkages in dextran. The ability of oligosaccharides to modify the idio type of this protein has allowed an independent measurement of the combining site size that confirms the findings of Cisar et al. (4) using inhibition of precipitation as an assay.

Antibodies bearing cross-reacting idiotypes that are modifiable by ligand have a high probability of binding the same antigen (5-7). The converse, however, is not true (7, 8) as will be discussed. This study provides further examples of this correlation by describing the relationship of idio type to the combining activities of mouse myeloma proteins that bind dextran or levan (4).

*Materials and Methods*

Ascites fluids from mice bearing myeloma tumors QUPC 52, UPC 102, UPC 10, and UPC 61 were obtained from Dr. Michael Potter, National Institutes of Health. Tumor MOPC 104E was originally obtained from Dr. Michael Potter and has been maintained at the Salk Institute. All other myeloma tumors originated from Dr. Melvin Cohn's tumor collection. The anti- $\alpha(1 \rightarrow 3)$  dextran myeloma proteins UPC 102 (IgA $\lambda$ ), MOPC 104E (IgM $\lambda$ ), and J558 (IgA $\lambda$ ) were purified on dextran B1355-polyacrylamide gel columns as previously described (9). The anti- $\alpha(1 \rightarrow 6)$  myeloma proteins W3129 (IgA $\kappa$ ) and W3434 (IgA $\kappa$ ) were purified on Sephadex G75 columns (Pharmacia Fine Chemicals Inc., Piscataway, N. J.).

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The antilevan myeloma proteins W3082 (IgA $\kappa$ ), Y5476 (IgA $\kappa$ ), and J606 (IgG3 $\kappa$ ) were isolated by methods previously described (10, 11). Ascites fluids containing proteins QUPC 52 (IgA $\kappa$ ), UPC 10 (IgG2a $\kappa$ ), and UPC 61 (IgA $\kappa$ ) were tested directly. The characteristics of these proteins are summarized in reference 4, Table I.

W3129 Fab' was prepared from purified W3129 protein by the methods described by Rudikoff et al. (12), with the following modifications: W3129 was alkylated with iodoacetic acid, pepsin digestion was carried out at pH 4.36, and the Fab' was isolated on a Biogel P100 column (Bio-Rad Laboratories, Richmond, Calif.).

Oligosaccharides were prepared by acid hydrolysis of dextran 20 (Pharmacia). 10 g of dextran 20 were hydrolyzed in 100 ml of 0.5 N HCl for 3 h at 100°C. The products were lyophilized and separated by gel filtration on a 4 × 200 cm Biogel P-4 column in water. The purity of each  $\alpha(1 \rightarrow 6)$ -linked oligosaccharide was confirmed by descending paper chromatography in ethylacetate-pyridine-water (5:3:2).

Using the method of Potter and Lieberman (13) anti-idiotypic sera were produced by immunization either of A/HeJ mice with purified W3082 or J558 protein or CE/J mice with purified W3129 protein. Animals were bled 6 wk after the first injection and the antisera were pooled. Anti-J558 and anti-W3129 sera were passed over Sepharose columns (Pharmacia) to which BALB/c serum was bound by the method of Wofsy and Burr (14). Pooled anti-W3082 serum was used directly.

Purified myeloma proteins were iodinated by the chloramine-T method of Greenwood et al. (15). Idiotypic cross-reactions were analyzed as previously described (9) using a solid-phase radioimmunoassay similar to that described by Askenase and Leonard (16). 1:500 dilutions of anti-idiotypic serum was used in all cases.

## RESULTS

*Effect of Hapten on the W3129 Idiotypic.*—The ability of different oligosaccharides to inhibit the binding of [<sup>125</sup>I]W3129 protein to anti-W3129 is shown in Fig. 1. This binding was inhibitable by the  $\alpha(1 \rightarrow 6)$ -linked disaccharide, isomaltose. No inhibition could be detected by either the  $\alpha(1 \rightarrow 4)$ -linked disaccharide, maltose (Fig. 1) or as previously shown the  $\alpha(1 \rightarrow 3)$ -linked disaccharide, nigerose (9). Of the  $\alpha(1 \rightarrow 6)$ -linked oligosaccharides tested (Fig. 1.) isomaltose was the least effective. Isomaltotriose was significantly more effective than isomaltose and about 90% inhibition of [<sup>125</sup>I]W3129 binding was achieved at the highest concentration tested. Isomaltotetraose was slightly more effective than isomaltotriose, and isomaltopentaose slightly more effective than the tetraose. No difference could be detected between isomaltopentaose and isomaltoheptaose.

The relative effect of oligosaccharides in W3129 Fab' was investigated (Fig. 2). As the inhibition of W3129 binding to anti-W3129 by Fab' was slightly less effective than by W3129 (Fig. 3) the idiotypic may have been altered during the preparation of the fragment. However, all Fab' molecules retained a determinant(s) that cross-reacted with native W3129 protein since binding of [<sup>125</sup>I]Fab' to anti-W3129 could be completely inhibited by W3129 (Fig. 3). Different  $\alpha(1 \rightarrow 6)$ -linked oligosaccharides inhibited [<sup>125</sup>I]W3129 Fab' binding to anti-W3129 in the following order: isomaltohexaose = isomaltopentaose >> isomaltotetraose > isomaltotriose >>> isomaltose (Fig. 2).

*Cross-Reactions between Myeloma Proteins with Similar Specificity.*—Three

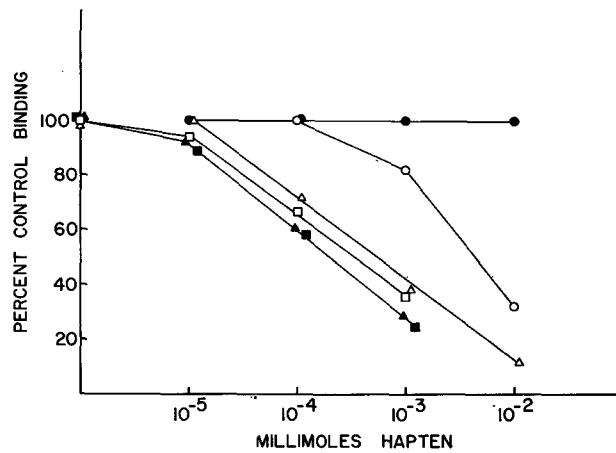


FIG. 1. Inhibition of  $[^{125}\text{I}]$ W3129 binding to anti-W3129 serum by oligosaccharides. Percent control binding = (cpm bound in the presence of hapten/cpm bound without hapten)  $\times 100$ . Each point represents the average of three determinations. The specific activity of the  $[^{125}\text{I}]$ W3129 was about 5,000 cpm/ng. The effects of the various oligosaccharides are represented as follows: ●, maltose; ○, isomaltose; △, isomaltotriose; □, isomaltotetraose; ▲, isomaltopentaose; ■, isomaltoheptaose.

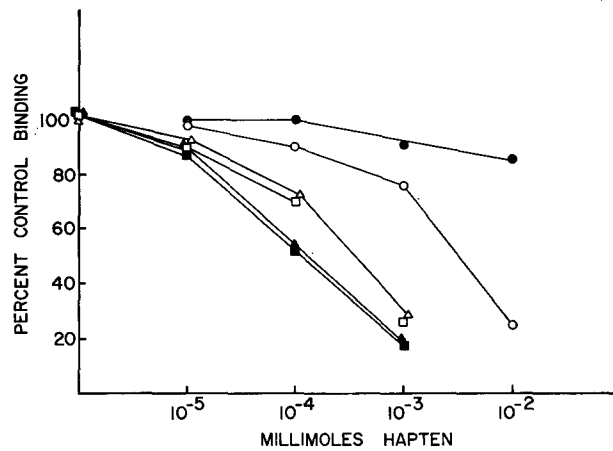


FIG. 2. Inhibition of  $[^{125}\text{I}]$ W3129 Fab' binding to anti-W3129 serum by oligosaccharides. Oligosaccharides are designated as in Fig. 1. Specific activity of the  $[^{125}\text{I}]$ W3129 Fab' was about 10,000 cpm/ng.

examples are shown here of cross-reactive idiotypic determinants found on myeloma immunoglobulins that share similar specificity.

(a) *Myeloma proteins with specificity for fructosans.* Five myeloma proteins with specificity for levan were tested for idiotypic cross-reaction. As shown in Fig. 4, considerable variation was found in the ability of these proteins to com-

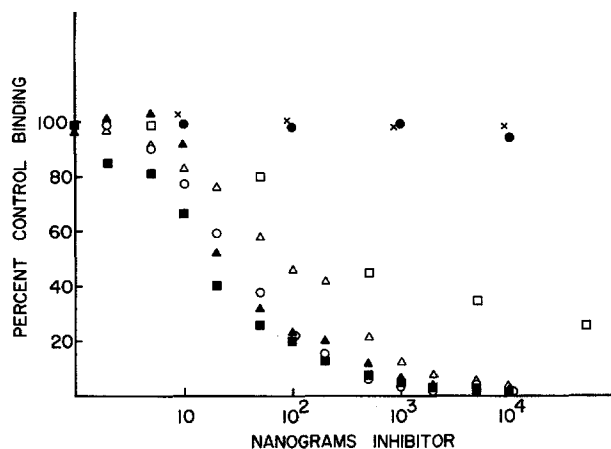


FIG. 3. Inhibition of [ $^{125}\text{I}$ ]W3129 and [ $^{125}\text{I}$ ]W3129 Fab' binding to anti-W3129 idiotypic serum. The inhibition of [ $^{125}\text{I}$ ]W3129 binding by various proteins are represented as follows: ●, J558; ×, QUPC 52; □, W3434; ○, W3129; and △, W3129 Fab'. Inhibition of [ $^{125}\text{I}$ ]W3129 Fab' binding is represented by ▲ for W3129 and ■ for W3129 Fab'. Percent control binding = (cpm bound in the presence of inhibitor/cpm bound without inhibitor)  $\times$  100. Each point represents the average of three determinations. Specific activities were the same as in Figs. 1 and 2. Protein concentrations were determined by the absorbance of undiluted protein solutions at 280 nm using an extinction coefficient of 1.40 per mg per ml, 1-cm light path, or in the case of QUPC 52 on the basis of the amount of protein precipitable by dextran from ascites fluid. The specific activity of [ $^{125}\text{I}$ ]W3129 used for QUPC 52 inhibition studies was about 1,500 cpm/ng.

pete with the binding of the antilevan protein, W3082, to anti-W3082 idiotypic serum. The ability of antilevan proteins J606 and UPC 10 to compete was not significantly better than proteins with unrelated specificities. Y5476 could completely inhibit [ $^{125}\text{I}$ ]W3082 binding but much less effectively than the W3082 protein. UPC 61 appeared somewhat more effective than W3082. However, the amount of antilevan protein present in the UPC 61 and UPC 10 ascites fluid was calculated as the amount of protein precipitable by levan in the ascites fluid from mice bearing these tumors (4). Hence the amount of inhibitor is a minimum estimate since nonprecipitable protein is not taken into account. This source of error as well as the possible overestimate of the W3082 protein concentration due to contaminating protein in the isolated W3082 protein preparation might explain why the UPC 61 protein appears more effective than W3082 in the inhibition assay. These two proteins, W3082 and UPC 61, might be identical.

(b) *Myeloma proteins with specificity for  $\alpha(1 \rightarrow 3)$  dextran.* Three myeloma proteins of this specificity were tested for idiotypic cross-reaction with J558. As previously shown cross-reaction was demonstrated between J558 and MOPC 104E, however, MOPC 104E inhibited considerably less effectively the [ $^{125}\text{I}$ ]-J558 binding to anti-J558 idiotypic serum (9). UPC 102 was also found to in-

hibit [ $^{125}$ I]J558 binding completely, however, less effectively than J558. As can be seen in Fig. 5, MOPC 104E and UPC 102 were indistinguishable as competitors of the [ $^{125}$ I]J558 binding, and therefore have similar idiotypes.

(c) *Myeloma proteins with specificity for  $\alpha(1 \rightarrow 6)$  dextran*: Idiotypic cross-

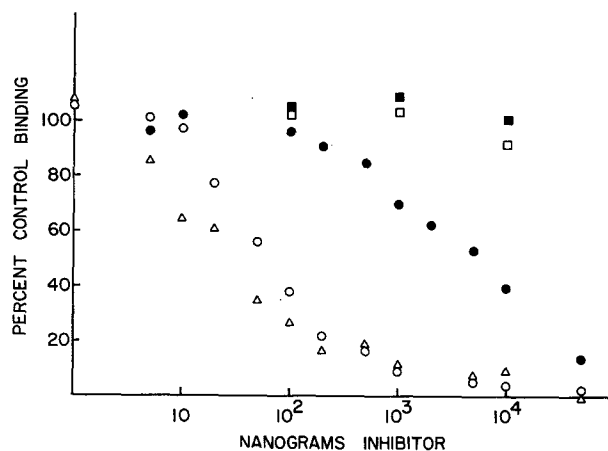


FIG. 4. Inhibition of [ $^{125}$ I]W3082 binding to anti-W3082 idiotypic serum. Inhibition by W3082, ○; UPC 61, △; Y5476, ●; J606, □; and UPC 10, ■. Percent control binding and protein concentrations were determined as described in Fig. 3 except for UPC 10 and UPC 61, the concentrations of which were based on the amount of protein precipitable by levan from ascites fluid. The points represent the average of three determinations. The specific activity of [ $^{125}$ I]W3082 was about 1,000 cpm/ng.

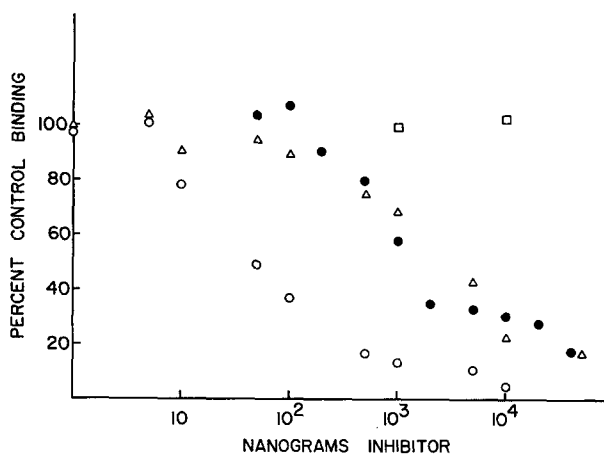


FIG. 5. Inhibition of [ $^{125}$ I]J558 binding to anti-J558 idiotypic serum. Inhibition by J558, ○; UPC 102, ●; MOPC 104E, △; W3129, □. Percent control binding and protein concentrations were determined as described in Fig. 3 legend. Each point represents the average of three determinations. The specific activity of [ $^{125}$ I]J558 was about 1,000 cpm/ng.

reaction was tested for three proteins that bind to  $\alpha(1 \rightarrow 6)$  linkages in dextran. As shown in Fig. 3, significant cross-reaction could be detected between W3129 and W3434 as compared to protein of unrelated specificity, J558. Complete inhibition of [ $^{125}$ I]W3129 binding to anti-W3129 was not achieved at the highest concentration of W3434 used. Therefore, the anti-W3129 idiotypic serum may recognize more than one determinant on the W3129 protein, not all of which the W3129 and W3434 proteins have in common. No cross-reaction could be detected between the W3129 protein and the anti- $\alpha(1 \rightarrow 6)$  protein QUPC 52.

#### DISCUSSION

The relationship between the idio type and the combining site is illustrated by these studies on the anti- $\alpha(1 \rightarrow 6)$  dextran protein, W3129, the anti- $\alpha(1 \rightarrow 3)$  dextran protein, J558, and the antilevan protein, W3082.

As shown by Cisar et al. (4) the precipitation of W3129 protein by dextran was specifically inhibited by oligosaccharides containing  $\alpha(1 \rightarrow 6)$  linkages. Studies on the inhibition of precipitation by  $\alpha(1 \rightarrow 6)$ -linked oligosaccharides of different lengths implied that the combining site of the W3129 antibody is most complementary to isomaltopentaose. Our studies on the inhibition of the W3129 protein and W3129 Fab' binding to anti-W3129 idiotypic serum by various oligosaccharides closely correspond to this finding because binding was specifically inhibited by  $\alpha(1 \rightarrow 6)$ -linked oligosaccharides and the order of inhibition by  $\alpha(1 \rightarrow 6)$ -linked oligosaccharides could be roughly described as isomaltotetraose = isomaltopentaose  $\gg$  isomaltotetraose  $>$  isomaltotriose  $\gg$  isomaltose. Both types of assay yielded similar results though isomaltotetraose was slightly more effective than isomaltotriose as an inhibitor of W3129 binding to anti-W3129 but indistinguishable from isomaltotriose as an inhibitor of dextran precipitation by W3129. Possibly the assay using ligand modulation of determinants is more discriminatory than that using inhibition of precipitation.

Three myeloma proteins (J558, MOPC 104E, UPC 102) that bind dextrans containing  $\alpha(1 \rightarrow 3)$  linkages have been shown to vary significantly in their apparent affinities. For example whereas inhibition by nigerose oligosaccharides of dextran precipitation by J558 indicated that the J558 combining site is of pentasaccharide size (17), MOPC 104E and UPC 102 by this assay have sites probably no larger than a trisaccharide (4, 18). As the binding of J558 to anti-J558 was at least 60% inhibitable even by nigerose, the oligosaccharide least effective in its ability to inhibit J558 precipitation of dextran, most if not all of the antibody to J558 is directed against a ligand-modifiable determinant. Differences in combining site should be expected, therefore, to be reflected in the idiotypes of these proteins. As shown here, though cross-idiotypic specificity could be detected, significant differences were apparent between J558 and MOPC 104E or UPC 102. UPC 102 and MOPC 104E were, however indistinguishable by this assay.

The idea that cross-idiotypic specificity implies cross-combining specificity is

further confirmed for the proteins that bind either levan or  $\alpha(1 \rightarrow 6)$  dextran. Inhibition of levan binding by  $\beta(2 \rightarrow 1)$ -linked fructose oligosaccharides indicated that W3082 and UPC 61 have indistinguishable combining sites (4) larger than J606 (17). W3082 and UPC 61 had indistinguishable idiotypes. In contrast, UPC 10 has specificity for  $\beta(2 \rightarrow 6)$ -linked fructose oligosaccharides (4). No cross-idiotypic specificity could be detected between W3082 and either J606 or UPC 10. Y5476 like UPC 10 also differs from W3082 by exhibiting greater specificity for  $\beta(2 \rightarrow 6)$ -linked fructose (4). However, in this case, a weak idiotypic cross-reaction could be detected between Y5476 and W3082. Of the proteins with specificity for  $\alpha(1 \rightarrow 6)$  dextran, W3129 and W3434 have combining sites most complementary to a pentasaccharide while QUPC 52 is most complementary to a hexasaccharide (4). No cross-idiotypic specificity could be detected between QUPC 52 and W3129. Cross-idiotypic specificity could be shown between W3434 and W3129 though they are clearly not identical. Further, at the highest concentration tested, W3434 did not completely inhibit W3129 binding to anti-W3129. Thus W3434 may not share cross-idiotypic specificity with all determinants recognized by anti-W3129.

This was not the case for either anti- $\alpha(1 \rightarrow 3)$  dextran or antilevan proteins with cross-idiotypic specificity; complete inhibition of the reference protein binding was achieved. The idio-type differences in these cases may result from modulations of the same determinant. Sequence analysis of these proteins should reveal those amino acid substitutions that determine these differences in idio-type.

In summary it is well to stress that the same criteria have not been used to describe the idiotypic determinants studied here as ligand modifiable. In the case of the W3129 protein, ligand modification was directly shown by the effect of ligand on the W3129 idio-type. For W3434 or UPC 102 and MOPC 104E such a relationship can be implied since these proteins have idiotypes that cross-react with reference proteins, W3129 and J558 (9) for which ligand modification of idio-type has been demonstrated. It has been assumed that ligand-modifiable determinants are being studied in the case of the antilevan proteins because of the concordance between specificity and idio-type, but it has not been shown directly because of the lack of suitable ligands.

The idiotypic ligand-modifiable determinant could be the combining site itself or a separate region subject to a conformational change resulting from an interaction of the combining site with the ligand. In either case, the inhibition of the idio-type-anti-idio-type reaction by different ligands will be related to the affinity of the antibody for them. As the assays using idio-type modification or inhibition of precipitation by different oligosaccharides yield similar results, it is not possible to determine whether the ligand-modifiable determinant is or is not the combining site itself. The only structural limitation imposed by our experiments in extension of earlier studies (19) is that the ligand-modifiable determinant be in the Fab' portion of the antibody. The probability, however, is that

it is confined to the light chain variable region ( $V_L$ )-heavy chain variable region ( $V_H$ ) domain as demonstrated for the idiotypic determinants of the antidi-nitrophenol myeloma protein, MOPC 315 (20).

Cross-idiotypic specificity has been observed between monoclonal human immunoglobulins (5, 6), between mouse myeloma proteins (7), and between mouse myeloma proteins and normal mouse antibodies (21). In these cases cross-idiotypic reactions have been found only among groups of proteins with similar combining specificities. However, the converse is not true. For example, antibodies binding phosphorylcholine need not show cross-idiotypic specificity (7). A further example of these relationships is provided by the studies on the myeloma proteins that bind different dextrans or levans. Antibodies that bind to a given antigenic determinant are found to have either indistinguishable idiotypes or similar but nonidentical idiotypes or noncross-reacting idiotypes. However, given cross-idiotypic specificity, cross-combining specificity is likely.

If a ligand-*modifiable* idotype is the combining site itself, this correlation between idotype and specificity is expected to be nearly perfect. If the ligand-modifiable determinant is distinct from the combining site but modifiable by conformational changes, a good correlation is still expected, because amino acid substitutions in the complementarity determining regions have been shown to alter idotype (9). The correlation in this case might be less perfect as some amino acid replacements in the complementarity determining regions (which presumably affect combining specificity) are not detectable by antisera to the ligand-modifiable determinants (9). This correlation is expected to be poorer for other so called "idiotypic" determinants, i.e., variable region determinants. One can imagine that a number of ligand-nonmodifiable "idiotypes" could be expressed either on  $V_L$  or  $V_H$  or as a result of a specific  $V_LV_H$  interaction. Such idiotypic determinants would be associated with antibodies of similar specificity with a probability dependent on the number of  $V_LV_H$  combinations the given antiserum distinguishes out of the total number expressed. On a somatic model, antisera recognizing ligand-nonmodifiable determinants would identify, in the extreme, all of the descendents of one germ line-encoded  $V_LV_H$  combination. What the specificities of the descendents would be, depends upon the starting sequence. Since this range is much smaller than the total, the association of a given specificity with a given idotype might appear to be unique but this would be misleading in the absence of a rather extensive survey with different antigens. Since amino acid substitutions that change the combining specificity of the antibody would not affect this class of idiotypic determinant, a given antiserum should recognize antibodies of unrelated specificity (22) with reasonable high frequency if the shared idiotypic determinant is ligand nonmodifiable.

Lastly, it should be pointed out that, with very low frequency it might be possible to find, in the case of ligand-modifiable determinants, cross-idiotypic specificity between antibodies of unrelated combining specificity. The finding of



rare exceptions to the rule would be of importance in analyzing the question as to whether conformational changes result from antibody interaction with ligand.

#### SUMMARY

This paper deals solely with idiotypic determinants, the configurations of which are modified when the antibody bearing them interacts with its ligand. This phenomenon is measured as an inhibition of the reaction between anti-idiotypic and idio type.

Two points are made: (a) The assay for ligand-modifiable determinants can be used to determine the "size" of the combining site. This is illustrated here with the anti- $\alpha(1 \rightarrow 6)$  dextran mouse myeloma immunoglobulin W3129. Whether the interaction between a homologous series of  $\alpha(1 \rightarrow 6)$  oligosaccharide ligands and the combining site of W3129 is measured by inhibition of precipitation with  $\alpha(1 \rightarrow 6)$  dextran (4) or of binding of W3129 to anti-W3129 idio type, the finding is the same. The order of inhibition is isomaltohexaose = isomaltopentaose  $\gg$  isomaltotetraose  $>$  isomaltotriose  $\gg\gg$  isomaltose. The combining site is optimally complementary to isomaltopentaose.

(b) Cross-idiotypic specificity is closely correlated with cross-combining specificity; the converse is not true. This is illustrated here with three groups of mouse myeloma immunoglobulin, each specific for  $\alpha(1 \rightarrow 3)$  dextran,  $\alpha(1 \rightarrow 6)$  dextran,  $\beta(2 \rightarrow 1)$  or  $\beta(2 \rightarrow 6)$  levan. If a given anti-idiotypic serum cross-reacted with several myeloma proteins, they always had similar combining specificity. Thus the three proteins, J558, MOPC 104E, and UPC 102, which cross-react with anti-J558 have combining specificity for  $\alpha(1 \rightarrow 3)$  dextran; cross-reacting W3082, UPC 61, and Y5476 have specificity for levan; and cross-reacting W3129 and W3434 have specificity for  $\alpha(1 \rightarrow 6)$  dextran. This extends previous studies with proteins specific for phosphorylcholine (7) or  $\gamma$ -globulin (8). As expected, the converse is not true, for proteins may have combining specificity for  $\alpha(1 \rightarrow 6)$  dextran e.g. QUPC 52, or levan e.g. J606, UPC 10 and yet not carry the above-mentioned reference idiotypes.

The correlation between cross-idiotypic and combining specificity breaks down when idiotypic determinants which are not modifiable by ligand are studied. The implications of this are pointed out since most investigations deal with ligand-nonmodifiable determinants.

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