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Reaction of Anti-idiotypic Antibody with the Hapten-Binding Site of a Myeloma Protein

(mice/plasma cell tumor/antigenic determinant/rabbit/Fab)

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ABSTRACT **Rabbit antiserum was prepared against mouse myeloma Protein-315, an IgA protein with specificiy toward the 2,4-dinitrophenyl group. After absorption of the antiserum with another IgA myeloma protein and with affinity-labeled Protein-315, the antiserum was specific for idiotypic determinants on Protein-315. Monovalent ligands that bind to Protein-315 with high affinity strongly inhibited the reaction of the protein with its anti-idiotypic antiserum. This indicated that the region of the hapten-binding site is a major idiotypic determinant. The myeloma protein is thus similar to rabbit antibenzoate antibody in this respect. These results, considered in conjunction with other data in the literature, indicate that an anti-idiotypic antiserum prepared in an isologous or heterologous species can recognize the same determinant, in this case the region comprising the ligand-binding site. Quantitative aspects of the data indicate that there is competition between the hapten and anti-idiotypic antibodies for the site.**

Monoclonal proteins, such as those associated with multiple myeloma (1), and populations of specific antibody from individual animals (2, 3) possess individually specific or "idiotypic" (4) antigenic determinants. Thus, some of the antibodies elicited by immunization with a myeloma protein will react exclusively with the immunogen. Idiotypic determinants on the immunoglobulin molecule have been localized to the Fab fragment (5, 6), which has a molecular weight of about 50,000 and contains one antigen-binding site. In the case of rabbit antibenzoate antibodies, the region of the active site was shown to be an important idiotypic determinant (7). Specific haptens markedly inhibited the reaction of rabbit anti-idiotypic antibody directed to the antibenzoate antibody molecule; there was a direct correlation between the inhibitory capacity of a hapten and its affinity for the antibody.

Many human and mouse myeloma proteins have recently been shown to bind certain antigens specifically. If the association constant for the binding is high, and if each Fab fragment has a single combining site, the inference is strong that the myeloma protein is an antibody with specificity for the antigen under investigation. It is always possible, however, that the reaction studied is a cross reaction, i.e., that the true specificity of the immunoglobulin molecule is directed to another, structurally related antigen.

Abbreviations: Dnp, 2,4-dinitrophenyl; MOPC, mouse plasma cell tumor induced with mineral oil.

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The experiments reported here were performed to ascertain whether the ligand-binding site of a homogeneous myeloma protein interacts with its anti-idiotypic antibody. For this purpose we utilized the mouse myeloma protein, secreted by mouse plasma cell tumor (MOPC-315) that was induced with mineral oil (Protein-315) (8)¶. This protein has combining sites that are, except for their uniformity, indistinguishable from those of conventionally prepared antibodies (9).

Our results indicate that the region comprising the ligand-binding site of Protein-315 is an important idiotypic determinant. Comparison of our data with those of Sirisinha and Eisen (10), who prepared anti-idiotypic antisera to Protein-315 in BALB/c mice, indicates that antibodies elicited in an isologous and a heterologous species are capable of recognizing the same idiotypic determinant, in this case the active site. It is also evident that the region of the active site can be an idiotypic determinant in a myeloma protein with antibody-like activity, as well as in an antibody. This further strengthens the view that the ligand-binding site of Protein-315 is a typical antigen-binding site.

MATERIALS AND METHODS

Protein-315 was obtained from Dr. H. N. Eisen, who isolated it as the mildly reduced and aminoethylated protein from the sera of mice bearing the tumor MOPC-315, generously provided by Dr. Michael Potter. The protein belongs to the IgA class and has light chains that are similar but not identical to lambda chains (11). Nearly all of the myeloma sera used for absorption or for testing were also obtained from Dr. Potter.

Antiserum to Protein-315 was prepared by injecting a rabbit intradermally in several locations with a total of 1 mg of the protein emulsified in Freund's complete adjuvant. This was repeated three times at 3-week intervals. Six weeks after the final inoculation, the antiserum reacted with Protein-315 in the Ouchterlony test and showed a strong spur over mouse myeloma Protein-460 (IgA-κ). The antiserum was absorbed by addition of sufficient whole myeloma serum containing Protein-460 to eliminate the reactivity of the absorbed antiserum with that protein in the Ouchterlony test. Further absorption was performed with affinity-labeled Protein-315 as described under *Results*.

Protein-315 was affinity-labeled with *N*-bromacetyl-*N'*-Dnp-ethylenediamine by the procedure of Haimovich *et al.*

¶ The tumor was produced by Dr. M. Potter in a mouse having genes derived from both the BALB/c and C57 strains.

(12). Labeling of Protein-315 with ^{125}I was done by the method of McConahey and Dixon (13), which uses chloramine-T. About 0.5 atom of iodine was incorporated per molecule of protein. The labeled protein was dialyzed until more than 98% of the radioactivity was precipitable by 5% trichloroacetic acid. In precipitin tests, a minimum of 2500 counts above background were recorded for each supernatant–precipitate pair.

The percentage of ^{125}I-labeled Protein-315 molecules bound to antibody was determined by indirect precipitation. The labeled protein (generally 0.1 μg) was mixed with a slight excess of the absorbed rabbit antiserum (15 μl of a 40:1 dilution in normal rabbit serum). An additional 5 μl of undiluted normal rabbit serum was added to provide a higher concentration of IgG. The rabbit IgG and complexes containing ^{125}I-labeled Protein-315 were then precipitated with goat antiserum to the Fc fragment of rabbit IgG, which had been absorbed with mouse IgG and whole BALB/c serum. In control experiments, rabbit antiserum to bovine IgG was substituted for rabbit antiserum to Protein-315. The percentage of radioactivity precipitated in such controls varied from 2 to 6%. The value for the control was subtracted from experimental data obtained in the same run. Each test contained 50 μg of ovalbumin, which was added first to reduce the adherence of radiolabeled protein to glass. Inhibitors, when used, were incubated with the ^{125}I-labeled protein for 1 hr at 37°C and pH 8 before the addition of the rabbit antiserum. Mixtures were incubated for another hour at 37°C, and goat antiserum to the Fc fraction was then added. Mixtures were then allowe to stand for a minimum of 4 hr at room temperature at which time a heavy precipitate had settled out. No significant differences were noted in several tests when the incubation was allowed to proceed for another 12 hr. Precipitates were washed three times and dissolved in 0.1 N NaOH. The dissolved precipitate and the combined supernatants were adjusted to the same volume and their radioactivities were determined to ascertain the fraction of ^{125}I-labeled Protein-315 precipitated. Experiments were performed in duplicate.

RESULTS

Fig. 1 shows the inhibition of indirect precipitation of ^{125}I-labeled Protein-315 by unlabeled Protein-315 and by the affinity-labeled protein. Rabbit anti-Protein 315 antiserum absorbed with the serum of mice bearing MOPC-460 tumor was used in these tests; this rabbit serum will be referred to as anti-Protein 315 (absorbed). As would be expected, unlabeled Protein-315 was capable of displacing completely the ^{125}I-labeled ligand from its antibodies. In contrast, the affinity-labeled protein was much less effective, displacing a maximum of 33% of the bound ^{125}I-labeled protein at the concentrations tested. This observation indicates that affinity-labeling results in the loss of one or more antigenic determinants. Since the only direct modification expected to occur upon affinity-labeling is in the ligand-binding site, it appeared that the site might be an antigenic, and presumably an idiotypic determinant. The possibility that a conformational change may also occur upon affinity labeling is considered in the *Discussion*.

To obtain further evidence relating to idiotypic specificity, the rabbit anti-Protein 315 (absorbed) was treated with affinity-labeled Protein 315 in a ratio of 5 μg of protein to 0.35 μl of the anti-Protein 315 (absorbed). As shown in Fig. 1, this represented an excess of the affinity-labeled protein over

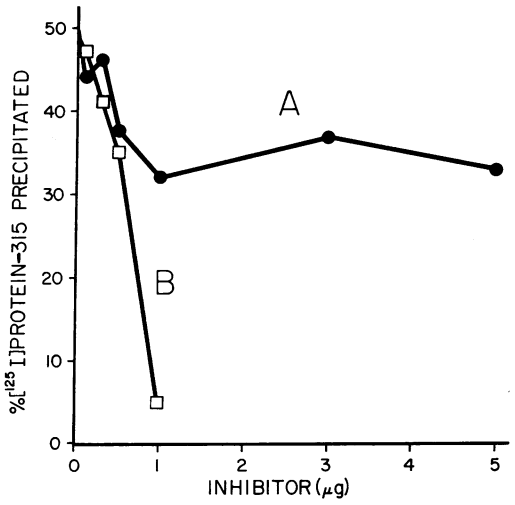

FIG. 1. Inhibition of binding of ^{125}I-labeled Protein-315 to rabbit anti-Protein 315 antiserum absorbed with Protein-460. Precipitation was performed by the indirect method. Each test comprised 0.1 μg of the labeled protein, 15 μl of a 40:1 dilution of anti-Protein 315 (absorbed), 5 μl of normal rabbit serum, and 0.2 ml of goat anti-rabbit fragment Fc. Unlabeled inhibitor was added to the antiserum before the labeled protein. Nonradioactive inhibitors: (*A*) ●——●, affinity-labeled Protein-315; (*B*) □——□, Protein-315. Experiments were performed in duplicate with average deviation from the mean, expressed as percent ^{125}I bound, of 1.5%.

the anti-Protein 315 antibodies. A group of 17 myeloma sera, (including Myeloma-315 serum), and normal sera from BALB/c, C57/BL, A/J, and DBA mice were tested for their capacity to displace ^{125}I-labeled Protein-315 from the rabbit antiserum that had been absorbed with Protein-460 and with affinity-labeled Protein-315. Myeloma 315 serum (0.5 μl) completely inhibited the binding of the labeled ligand. Ten times this volume of each normal serum had no inhibitory activity. The 17 other myeloma sera tested, in 5 μl quantities, included 11 IgA-κ (kappa light chain), 1 IgA-λ (lambda light chain), 2 IgG-κ, 1 IgH-κ, and 2 IgF-κ. One of the myeloma sera with an IgA-κ protein (MOPC-511) also contained free λ chains. Of these 17 myeloma sera, one (RPC 6A, IgA-κ) gave 47% inhibition and two (MOPC-511, IgA-κ and Mc 603, IgA-κ) caused 40% inhibition. The remaining sera gave 30% inhibition or less. It should be noted that the weight ratio of unlabeled immunoglobulin to labeled Protein-315 was very high in these experiments. If we assume a protein concentration of 10 mg/ml or more in the myeloma sera, the minimum ratio was 500:1. The results indicate that the antiserum absorbed with Protein-460 and affinity-labeled 315 interacts with idiotypic determinants.

Evidence that the major idiotypic determinant recognized by doubly absorbed antiserum to Protein-315 is the combining site

If the only modification brought about by affinity-labeling is in the active site, then absorption of anti-Protein 315 serum with the affinity-labeled protein should leave only antibodies reactive with the ligand-binding site of Protein-315. However, in view of the possibility that affinity-labeling might modify the protein in some other manner, additional proof was required. Such evidence was sought by measurement of the effect of mixing ^{125}I-labeled Protein-315 with specific haptens

TABLE 1. *Effect of haptens on the binding of ^{125}I-labeled Protein-315 by its absorbed rabbit antiserum**

Haptens	Concentration of hapten† (mol/liter)	^{125}I-labeled Protein-315 Precipitated (% of control‡)
ε-2,4-Dnp-L-lysine	6×10^{-3}	28 ± 1
	3×10^{-3}	40 ± 5
	1.2×10^{-3}	45 ± 1
	3×10^{-4}	66 ± 5
ε-2,4-Dnp-caproate	6×10^{-3}	22 ± 1
	3×10^{-3}	33 ± 2
	1.2×10^{-3}	46 ± 1
	3×10^{-4}	62 ± 2
ε-Aminocaproate§	6×10^{-3}	82 ± 5
2,4-Dinitrophenol	6×10^{-3}	92 ± 0
	3×10^{-3}	94 ± 1
p-Nitrobenzoate	6×10^{-3}	112 ± 2
p-Nitrophenol§	6×10^{-3}	111 ± 5

* Absorbed with Protein-460 and with affinity-labeled Protein-315 (5 μg/0.35 μl) as described in the text. Precipitation was performed by the indirect method. In the absence of inhibitor, 26% of the ^{125}I-labeled Protein-315 was precipitated after the double absorption. Experiments are in duplicate with average deviations as shown.

† Concentration before addition of goat anti-rabbit Fc.

‡ The amount of radioactivity bound by doubly absorbed antiserum is taken as 100%.

§ For this experiment the rabbit antiserum was absorbed with a separate batch of affinity-labeled Protein-315, which displaced about 60% of the bound ^{125}I-labeled Protein-315.

before the addition of the doubly absorbed rabbit antiserum to Protein-315. Haptens that are bound strongly by Protein-315 might be expected to prevent combination of ^{125}I-labeled Protein-315 with antibodies specific for the ligand-binding site. The results are shown in Table 1. Of the haptens tested, only ε-2,4-Dnp-L-lysine and ε-Dnp-aminocaproate strongly inhibited binding by the anti-Protein 315 antiserum that had been absorbed with Protein-460 and with affinity-labeled Protein-315. Each of the two haptens caused more than 70% inhibition of binding at a concentration of 6×10^{-3} M‖. These are also the only compounds of the six tested that would be expected to combine with Protein-315 with high affinity. ε-Aminocaproate, *p*-nitrophenol, and *p*-nitrobenzoate lack the 2,4-dinitro group recognized by Protein-315; 2,4-dinitrophenol combines with low affinity (14), presumably owing to the absence of a hydrocarbon side chain.

DISCUSSION

After absorption with serum containing myeloma Protein-460 (IgA-κ) and with an excess of affinity-labeled Protein-315, rabbit anti-Protein 315 antiserum appeared to be specific for idiotypic determinants. This was indicated by the failure of normal sera from several strains of mice (BALB/c, C57/BL, DBA and A/J) to inhibit the binding of ^{125}I-labeled Protein-315 by the absorbed antiserum. Of 17 myeloma sera tested, three showed significant inhibition, but the largest value was less than 50% despite the very high ratio of unlabeled to labeled protein in each test. In a volume 10% as large myeloma serum-315 completely displaced ^{125}I-labeled Protein-315 from the doubly absorbed antiserum.

It might have been difficult to obtain an antiserum specific for idiotypic determinants without the use of affinity-labeled protein since Protein-315 contains light chains of a subtype closely related to lambda chains, which has not yet been found in any other myeloma protein or in normal mouse sera (11).

In its reaction with the antiserum absorbed only with Protein-460, affinity-labeled Protein-315 was much less effective as an inhibitor than unlabeled Protein-315 (Fig. 1). At a weight ratio of 10:1, unmodified, unlabeled Protein-315 displaced nearly all of the ^{125}I-labeled Protein-315 from its rabbit antibody, as might be expected (Fig. 1). These results can be explained on the basis that affinity-labeled and unmodified Protein-315 share some, but not all antigenic determinants that are recognized by the rabbit antiserum after absorption with Protein-460. This finding and the relatively weak interaction of other myeloma proteins with absorbed anti-Protein 315 antibodies indicate that a large proportion of these antibodies are specific for idiotypic determinants.

Since chemical modification by the affinity-label occurs in the active site, this result indicates either that the active site itself is an idiotypic determinant or that affinity-labeling causes a conformational change outside the site, resulting in the loss of an antigenic determinant. If the active site is an idiotypic determinant, and if affinity-labeled Protein-315 lacks only this determinant, one would predict that a combination of affinity-labeled Protein-315 and specific hapten might completely inhibit the reaction of ^{125}I-labeled Protein-315 with its anti-idiotypic antibodies. The hapten should block the active site of the labeled Protein-315 and thus prevent its interaction with those remaining antibodies that had not combined with the excess of affinity-labeled protein present.

A similar result would be predicted if affinity-labeling induces a conformational change, provided that the free hapten causes a similar change in the native molecule. Suppose that affinity-labeling introduces a conformational change and causes an alteration in an idiotypic determinant, which we will designate D, such that anti-D antibodies will no longer combine with this idiotypic site. Such an alteration would of course account for the failure of affinity-labeled Protein-315 to displace all of ^{125}I-labeled Protein-315 from its anti-idiotypic antibodies; the ^{125}I-labeled protein would still combine with anti-D antibodies. However, if hapten were now added to the system, it would combine with the native ^{125}I-labeled protein and, if it induced the same conformational change, the hapten–protein complex would no longer be bound by anti-D. Thus, a combination of affinity-labeled protein and hapten should completely displace the ^{125}I-labeled protein under these circumstances. That affinity-labeling of Protein-315 introduces the hapten in such a way that it occupies the combining site in a position very similar of that of free hapten is indicated by the results of Givol *et al.* (15).

The results in Table 1 indicate that a combination of free hapten and affinity-labeled Protein-315 are indeed capable of displacing nearly all of the ^{125}I-labeled protein from its absorbed antibody. When haptens that bind specifically to Protein-315 were used in conjunction with the affinity-labeled protein, up to 78% displacement of the ^{125}I-labeled protein re-

‖ This concentration is calculated on the basis of the volume of solution before addition of goat antiserum to Fc; after the addition it was about 40% lower.

sulted. Molecules lacking the dinitrophenyl group, or dinitrophenol, which combines with low affinity (14), were relatively ineffective.

On a quantitative basis ε-2,4-Dnp-L-lysine and ε-2,4-Dnp-caproate were about equally effective as inhibitors. This correlates with the fact that they combine with almost equal affinity with Protein-315 (8). It is of interest that at a lower concentration, 3×10^{-4} M, ε-2,4-Dnp-L-lysine and ε-2,4-Dnp-caproate inhibited binding by the absorbed antibodies to only a small extent. Since the association constant for each hapten is about $10^7\ M^{-1}$ (8), the fraction of the combining sites of Protein-315 occupied by hapten in the absence of any anti-idiotypic antibody would exceed 99.9%. The fact that inhibition of precipitation was incomplete suggests that there is competition between hapten and anti-idiotypic antibody for the ligand-binding site of Protein-315. The same conclusion was drawn on the basis of the inhibition by haptens of the binding of rabbit anti-idiotypic antibodies to rabbit anti-benzoate antibody (7).

Because of the considerations discussed above, our data cannot distinguish between direct steric interference by the affinity-label or an induced conformational change. The same problem in interpretation arose in studies of $F(ab')_2$ fragments of antibenzoate antibodies (7). In that instance, it was shown that the combination with hapten affected the interaction with anti-rabbit Fab to only a small extent and had no effect on binding to antiallotype antisera. This indicated that if a conformational change does occur it is greatly restricted in its extent.

The results reemphasize the importance of the region of the antigen-binding site as a potential antigenic determinant. It seems reasonable in retrospect that this part of the molecule might be a significant idiotypic determinant. It must comprise residues of the hypervariable regions of the antibody molecule and should have unique contours to accommodate the antigen. The hydrophobic nature of certain antibody-combining sites (16) may also contribute to immunogenicity. Although the available data are not sufficient to establish the generality of this finding, it seems significant that specific haptens inhibit reactions with anti-idiotypic antisera in the two systems studied so far.

These and earlier data indicate the necessity of working with ligands of high affinity in order to demonstrate that the active site is an antigenic determinant. It is also apparent that an assay that measures binding rather than precipitation should be used. Precipitation must involve multiple antigenic determinants and blocking of one determinant by the ligand might not result in inhibition. In addition, it is essential to avoid the use of an excess of anti-idiotypic antibody in binding tests. Under such circumstances blocking of one determinant, the active site, by hapten would not diminish binding mediated by other idiotypic determinants. Only if the amount of antiantibody present is limiting would the inactivation of a single antigenic determinant be expected to diminish the percentage of antigen molecules bound.

It should be noted that the antigen-binding site is certainly not the only potential idiotypic determinant in an antibody molecule. This is shown by the fact that haptens do not completely inhibit the reaction of antibenzoate antibodies with their anti-idiotypic antibodies (7) and also by the observation that Fab fragments sometimes (5, 6) [although not always (17, 18)] form precipitates with anti-idiotypic antisera; precipitation of course requires antigenic multivalence. The results of Sirisinha and Eisen (10) suggest, however, that an anti-idiotypic antiserum raised within the isologous strain of mouse may recognize the antigen-binding site of the immunogen as the only significant idiotypic determinant.

Consideration of our data and those of Sirisinha and Eisen indicates that antisera to a myeloma protein produced in an isologous or a heterologous species may recognize the same idiotypic determinant, in this case the region of the ligand-binding site of the myeloma protein. This finding should tend to minimize the distinction between idiotypic (4) and individually specific (1) antigenic determinants that are defined through their recognition by antisera produced in the same or a different species, respectively. The observation that the region of the ligand-binding site of a myeloma protein, as well as that of rabbit antibenzoate antibody, is an idiotypic determinant provides additional support for the concept that a myeloma protein is a homogeneous but otherwise typical immunoglobulin.

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