Role of Antibody Valency in Hapten-Heterologous Immunoassays

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We studied the effects of hapten heterology on immunoassays of triiodothyronine (T3), digoxin, and cortisol, in a format involving labeled monoclonal antibodies and immobilized, protein-conjugated ligands. Replacing the homologous conjugated ligands T3, digoxin, and cortisol with their respective analogs diiodothyronine, digitoxin, and corticosterone led in each case to a decrease in the midpoint of displacement (ED50) for the same zero-dose signal. The mechanism of this phenomenon was studied by converting the bivalent anti-T3 to a monovalent whole antibody (bispecific monoclonal anti-T3 × anti-glucose-6-phosphate dehydrogenase) by cell fusion. The monovalent antibody was effective as a tracer in the homologous T3 assay, but generated a very low zero-dose signal with the heterologous solid phase, thus precluding sensitivity enhancement. On the basis of these results and additional kinetic and double-labeling experiments, we propose that the use of hapten heterology relies on bivalent binding of the antibody to the solid phase to compensate for a lower intrinsic affinity. This binding mechanism leads to lower assay concentrations of the ternary complex analyte–labeled antibody–immobilized hapten, thereby providing enhanced sensitivity.

Indexing Terms: triiodothyronine · digoxin · cortisol · monoclonal antibodies · bispecific antibodies · variation, source of

Hapten heterology, the structural difference between the hapten–protein conjugate used for immunization and assay reagent, is often incorporated into the design of competitive immunoassays as a means for improving assay sensitivity (1–7) or reagent stability (8). Hapten heterology is superior to bridge and site heterologies because it involves highly defined epitope changes, thus allowing better control over the reagents in practical immunoassays, and more accurate interpretation of results in basic immunochemical studies. In a previous study on solid-phase immunoassays of thyroid hormones, we found that hapten heterology can lower the displacement midpoint (ED50) of the reaction without lowering the zero-dose signal to the value expected from the intrinsic affinities of the antibodies. On the basis of mathematical modeling and failure to obtain this effect with either acridinium ester-labeled Fab fragment or 125I-labeled iodothyronines as the tracers, we concluded that cooperativity of tracer/solid-phase binding is a prerequisite for the beneficial effect of hapten heterology (6). In the present study we attempted to confirm and further define the nature of the cooperative binding in the 3',3,5-triiodothyronine (T3) immunoassay, and to determine whether the same mechanism operates in immunoassays for other analytes.2

Materials and Methods

Preparation of monoclonal antibodies. Monoclonal antibodies were prepared essentially as previously described (9). Preparation and assay performance of the anti-T3 monoclonal antibody have been reported (6). The monoclonal antibody against cortisol was prepared by using glucose-6-phosphate dehydrogenase (G6PDH)–3-O-carboxymethylxime (CMO)–cortisol as the immunogen. The anti-digoxin monoclonal antibody was purchased from Chemicon International, Inc., Temecula, CA.

Preparation of labeled and immobilized reagents. Monoclonal antibodies were purified by Protein A chromo-
matography with an Affi-gel Protein A MAPS II kit (Bio-Rad Labs., Richmond, CA) and labeled with the acridinium ester (AE) derivative 2',6'-dimethyl-4'-(N-succinimidyl-oxycarbonyl)-phenyl-10-methylacridinium-9-carboxylate methosulfate, as previously described (10). Bovine gamma globulin (BGG) was derivatized with hapten and then immobilized on paramagnetic particles (PMP) by the glutaraldehyde method (11). Derivatization of BGG with T₃ and 3,5-diiodothyronine (T₂) was previously described (6). Digoxin and digitoxin were coupled by the periodate oxidation method (12). Bovine serum albumin (BSA) conjugates of 3-CMO-cortisol and 3-CMO-corticosterone were purchased from Steraloids Inc., Wilton, NH. ¹²₅I-labeled T₉, digoxin, and cortisol tracers were obtained from their respective Magic RIA kits (Ciba Corning Diagnostics Corp., Walpole, MA). ¹²₅I was measured in a 400 Multi-Well Gamma Counter (Ciba Corning Diagnostics Corp.) and the results were expressed as counts per minute.

Preparation and characterization of the bispecific antibody. Hybridoma cell lines secreting bispecific monoclonal antibodies were prepared according to a published method (13). Briefly, a hypoxanthine/aminopterin/thymidine-sensitive cell line secreting anti-T₃ [same antibody as described in (6)] was selected by resistance to azaguanine and was cloned by limiting dilution. This cell line was used as a fusion partner with spleen cells from A/J mice immunized with G6PDH. Supernates from hybridoma cultures were screened for antibody activity by using AE-labeled G6PDH, [¹²⁵I]T₉, and PMP-immobilized goat anti-mouse IgG. Cells from wells producing antibody having both specificities were cloned twice by limiting dilution to ensure monoclonality of the hybridoma. A stable clone was injected into pristane-primed mice, and ascites was collected 10 days later. The immunoglobulin fraction of ascites was isolated by chromatography on Protein A.

Agarose gel electrophoresis (High Resolution Protein Electrophoresis; Ciba Corning Diagnostics Corp.) revealed three bands: one with migration similar to that of the anti-T₃ of the fusion partner and two more acidic ones. The bispecific antibody was purified from the mixture by two sequential affinity chromatographic steps with Sepharose 6B-immobilized BGG-T₉ and G6PDH (CNBr-activated Sepharose 6B was purchased from Pharmacia, Piscataway, NJ, and used according to the manufacturer's instructions). Antibody was eluted with 100 mmol/L glycine, pH 2.5, and the purified antibody was then dialyzed against 50 mmol/L sodium phosphate buffer, pH 7.4. The electrophoretic mobility of the affinity-purified antibody was found to coincide with the middle band.

Isotype determination by Mouse Isotyper kit (Bio-Rad Labs.) revealed both γ₂a, as in the parent anti-T₃, and γ₁ heavy chains. The bispecificity of the antibody was confirmed by its ability to bridge between [¹²⁵I]T₉ and PMP- or microtiter plate-immobilized G6PDH. This antibody was labeled with AE and used as the monovalent anti-T₃ tracer in the experiments reported in Results. The relative affinities to unconjugated T₃ and T₂ were determined by comparing the ED₅₀ values of T₃ and T₂ in immunoassays performed with the bivalent or monovalent labeled monoclonal antibodies. Relative affinity of the monovalent antibody to T₃ and T₂ was identical (0.4:100) to that of the bivalent antibody.

As an alternative procedure for purifying the bispecific antibody, we attempted ion-exchange on diethylaminoethyl-Sepharose, which separated the three original IgG bands. AE-labeling of these bands and testing their behavior as tracers in T₃ immunoassays revealed tracer activity only in the most basic band and the intermediate band. Interestingly, only 50% of the labeled intermediate band could be bound to excess PMP-immobilized BGG-T₉, suggesting the presence of inactive species. Also, the labeled basic band behaved in a T₃ assay somewhat like the bispecific antibody, suggesting the presence of monovalent anti-T₃ in the basic band. These observations are in accordance with at least partially random association of the heavy and light chains, as previously observed (13).

Immunoassays. For the conventional incubation protocol, we vortex-mixed the serum standards (50, 100, and 0 µL for the T₉, digoxin, and cortisol assays, respectively) for ~15 s with 0.1 mL of labeled antibody, added 0.5 mL of PMP suspension, and incubated the mixtures for 4 h at room temperature. The buffer for the T₉ assay contained, per liter, 20 mmol of sodium phosphate, 20 mmol of sodium barbital, 160 mmol of sodium chloride, 1 mmol of EDTA, 1 g of sodium azide, 150 mg of 8-anilino-1-naphthalene sulfonic acid, and 1 g of BSA at pH 7.4. The buffer for digoxin and cortisol assays contained, per liter, 50 mmol of sodium phosphate, 150 mmol of sodium chloride, 1 mmol of EDTA, 1 g of sodium azide, and 1 g of BSA (pH 7.4).

To assess the effect of delayed addition of PMP, we incubated the standards for 3.5 h with the same amount of labeled antibody in 0.5 mL of buffer, then added concentrated PMP in 0.01 mL, and continued the incubation for an additional 0.5 h. For the simultaneous incubation protocol, we mixed 0.01 mL of PMP with the standards, added 0.5 mL of labeled antibody, and incubated for 4 h. After the incubation we washed the solid phases and read their chemiluminescence in the luminometer (Magic Lite Analyzer II; Ciba Corning Diagnostics Corp.), expressed as relative light units (RLU) as previously described (14). All assays were run in triplicate and exhibited CVs of <5%. CVs of ED₅₀ determinations from three experiments were <10%.

Results

Immunoassays with bivalent antibodies. We first generated displacement curves of T₉, digoxin, and cortisol assays with whole (bivalent) monoclonal antibodies, using the 4-h conventional incubation protocol. We optimized the concentrations of the labeled antibodies and solid phases to obtain maximal sensitivity, using the homologous combinations. To assess the effect of heterology, we then substituted the heterologous solid phases for the homologous ones, at concentrations that yielded the same zero-dose signal (Figure 1). The mag-
The AE-labeled antibodies and solid phases were at the same final assay concentrations as for Fig. 1. For simultaneous incubation, the solid phases were added in 0.01 mL to the standards before adding 0.5 mL of labeled antibodies, followed by 4 h incubation. For delayed addition of solid phase, the standards were incubated for 3.5 h with 0.5 mL of labeled antibodies, followed by addition of 0.01 mL of solid phases and 0.5 h additional incubation. Signals at zero dose were not affected by heterology, but were lowered approximately twofold by delayed addition.

Table 1. Effects of Hapten Heterology and Delayed Solid-Phase Addition on Midpoints of the Displacement Curves

<table>
<thead>
<tr>
<th>Solid phase</th>
<th>Simultaneous addition</th>
<th>Delayed addition</th>
</tr>
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<tbody>
<tr>
<td>PMP-BGG-T3</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>PMP-BGG-T2</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>PMP-BGG-digoxin</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>PMP-BGG-digitoxin</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>PMP-BSA-cortisol</td>
<td>450</td>
<td>220</td>
</tr>
<tr>
<td>PMP-BSA-corticosterone</td>
<td>340</td>
<td>100</td>
</tr>
</tbody>
</table>

The AE-labeled antibodies and solid phases were at the same final assay concentrations as for Fig. 1. For simultaneous incubation, the solid phases were added in 0.01 mL to the standards before adding 0.5 mL of labeled antibodies, followed by 4 h incubation. For delayed addition of solid phase, the standards were incubated for 3.5 h with 0.5 mL of labeled antibodies, followed by addition of 0.01 mL of solid phases and 0.5 h additional incubation. Signals at zero dose were not affected by heterology, but were lowered approximately twofold by delayed addition.

Fig. 1. Displacement curves prepared with bivalent AE-labeled antibodies in our conventional incubation protocol: (A) T3 assay with PMP-BGG-T3 (○) and PMP-BGG-T2 (□); (B) digoxin assay with PMP-BGG-digoxin (○) and PMP-BGG-digitoxin (□); (C) cortisol assay with PMP-BSA-cortisol (○) and PMP-BSA-corticosterone (□)

Fig. 2. Displacement curves for T3 assay prepared with a monovalent (bispecific) AE-labeled antibody

The curves for PMP-BGG-T3 (○) and PMP-BGG-T2 (□) were obtained under incubation conditions and reagent concentrations identical to those described for Fig. 1A.

The signal to the amount obtained by the homologous solid phase required a 30-fold increase in the concentration of the heterologous solid phase; however, this led to a further increase in ED50. These results cannot be explained on the basis of intrinsic affinities, because the monovalent monoclonal had an unaltered ratio of affinities to T2 and T3 (0.4:100). Therefore, the results strongly suggest dependence on bivalent association for effective antibody–heterologous solid-phase interaction.

Ability of bivalent antibody to bridge labeled and immobilized ligands. Additional evidence in support of the role of bivalent association in heterology was obtained by a double-label approach. We incubated AE-labeled bivalent antibodies with various solid phases as in the immunoassays, except that trace concentrations of 111I-labeled analytes (~50,000 counts/min) were included. At the end of 4 h of incubation, the amount of bound tracer remaining on the washed PMPs was read by both the gamma counter and the luminometer.

Antibody binding to PMP, assessed by chemiluminescence,
was not affected by the presence of $^{125}$I-labeled haptens. Binding of radiolabeled haptens was generally low and not detectable in the cross-iso assay. However, in the $T_s$ and digoxin assays, binding of radiolabeled hapten to homologous MPM was greater than in the heterologous assay by fourfold (402 bound counts/min per bound $10^6$ RLU vs 91 bound counts/min per bound $10^6$ RLU) and twofold (187 bound counts/min per bound $10^6$ RLU vs 77 bound counts/min per bound $10^6$ RLU), respectively. The background radioactivity range was 10–22 counts/min and the CVs were <10%. These results suggested that in the heterologous combinations a higher fraction of the antibody molecules had both their arms occupied with immobilized hapten.

**Discussion**

In a previous investigation we noted that a labeled anti-$T_s$ bound strongly to either immobilized $T_s$ or $T_2$, whereas its Fab fragment bound strongly to immobilized $T_3$, but not to $T_2$. To explain these findings, we suggested a mechanism that involves positive binding cooperativity with the solid phase. However, because Fab fragments differ from the whole antibody in several structural features, it was not clear whether the avidity associated with the whole antibody resulted from bivalent binding or from non-specific protein–solid-phase interaction (6).

In the present work a highly defined monovalent whole antibody behaved like the Fab fragment, thus supporting a mechanism involving specific bivalent association. Additional support for this mechanism emanates from our observation of higher $^{125}$I-labeled hapten binding capacity of the antibodies when bound to immobilized homologous (vs heterologous) ligands, because this implies a higher fraction of free binding arms, or a greater stability of the ternary complexes.

Mathematical modeling by Rodbard and Weiss (15) of competitive immunoassays utilizing labeled antibodies led to the prediction that antibody bivalence reduces sensitivity by a factor of two (when antibody concentration is adjusted to the reciprocal of the affinity constant to obtain maximal sensitivity). This prediction stems from the ability of the bivalent antibody to bind to the solid phase with one binding site, even when its other binding site is occupied by an analyte molecule. In the heterologous assay, this twofold loss of sensitivity is expected to be at least partially avoided, because formation of the ternary complex is suppressed. An additional twofold loss of sensitivity in competitive immunoassays results from the equilibration of the antibody with the immobilized antigen or the labeled analyte, depending on the format, because their concentrations are also in the vicinity of the reciprocal of the affinity constant. This latter loss of sensitivity can be at least partially avoided in both assay formats by “delayed addition” of the solid phase or the labeled analyte, as described by Rodbard et al. (16). The realization of this effect in our study, regardless of whether the assays were homologous or heterologous, strongly suggests that the effect of heterology does not involve overcoming this source of sensitivity loss. Combining the two effects is expected to lead to no more than fourfold-improved sensitivity, as observed in this study. The proposed mechanism is consistent with another advantage of heterologous assays, namely, a markedly reduced susceptibility to inhibition by ligands leaching from the solid phase to the aqueous medium; the result is greater reagent stability (7, 8, 17).

Although we studied the digoxin and cortisol assays less extensively, these assays responded to heterology and delayed addition of solid phase similarly to the $T_3$ assay, suggesting a common mechanism. This mechanism probably extends to all competitive immunoassays, regardless of whether the assays are configured with hapten-, bridge- or site-heterology; furthermore, the equivalent mechanism is probably involved in heterologous immunoassays configured with immobilized antibody and labeled multivalent analytes. In the present work the monovalent antibody was produced bio-synthetically, without resorting to chemical or enzymatic modifications. Further application of highly defined and customized antibodies and antigens, recently made available by advances in cell and molecular biology, should facilitate future investigations on the role of valency in immunochemical reactions.

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**References**


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Assessment of Copper Status: Effect of Age and Gender on Reference Ranges in Healthy Adults

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We measured major indices related to copper nutritional status in 55 men and 86 women between ages 20 and 83 years who were in apparent good health. Plasma copper concentrations and both immunoreactive and enzymatically measured ceruloplasmin were significantly higher in women than in men and were higher in women taking oral contraceptives. Plasma copper, immunoreactive ceruloplasmin, and cytochrome-c oxidase in platelets and mononucleated leukocytes tended to increase with age. The ratio of enzymatic to immunoreactive ceruloplasmin, erythrocyte superoxide dismutase, and 67Cu uptake by erythrocytes were not significantly affected by either age or gender. Thus, factors other than copper nutriture—such as age, gender, and hormone use—need to be considered when using many of these indicators to evaluate copper nutritional status.

Indexing Terms: ceruloplasmin · superoxide dismutase · cytochrome-c oxidase · nutritional status · variation, source of

The nutritional essentiality of copper is well established, and nutritional copper deficiency can occur in humans under a variety of conditions. Dietary copper deficiency has been described in premature infants, neonates, and previously malnourished children (1–3) and results in anemia, leukopenia, and skeletal demineralization. Similar symptoms have been described in patients after long-term total parenteral nutrition with copper-deficient alimentation solutions (4).

Klevay has suggested (5) that subclinical copper depletion contributes to an increased risk of heart disease through instability of heart rhythm and hyperlipidemia. This concept is supported both experimentally (6, 7) and epidemiologically (8). However, it has been difficult to demonstrate that copper depletion occurs in adult populations by conventional assays of copper status. This was particularly evident in several studies of experimental copper depletion of men and women, in whom physiological changes, such as abnormal electrocardiograms (9, 10), abnormal glucose tolerance (11), and blood pressure changes (12) were noted but conventional biochemical signs of copper depletion were largely absent or inconsistent (9–13).

Here we discuss several proposed indices for assessing copper nutritional status, reference ranges, and the effects of age, gender, and estrogen use on these indices as part of a study (14) on the effects of age and sex on copper absorption, biological half-life, and status.

Materials and Methods

Subjects

Fasting blood samples were collected from apparently healthy free-living subjects, 55 men and 86 women, who were volunteers in a study on the effects of age and sex on copper absorption and rate of excretion that was carried out at the Grand Forks Human Nutrition Research Center. The volunteers entered the study after they had been informed in detail of the nature of the research, and after it was established that they were healthy, nonpregnant, nonsmokers and were not taking vitamin or mineral supplements or prescribed medications. The exceptions were that some women between ages 20 and 39 were taking oral contraceptives and some women over 50 were on estrogen replacement therapy. This study was approved by the Institutional Review Boards of the University of North Dakota and the US Department of Agriculture and followed guidelines of the Department of Health and Human Services and the Helsinki Doctrine regarding the use of human subjects.

Analytical Methods

Blood was drawn into plastic syringes from an antecubital vein, which had been made visible by temporary use of a tourniquet, after the subjects had fasted for 12 h. Aliquots were mixed with the appropriate anticoagulants and processed within 90 min of the time the blood was drawn. Plasma copper concentration was deter-

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