Antigen-Binding Site Protection During Radiolabeling Leads to a Higher Immunoreactive Fraction

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It is generally accepted that the immunointegrity of an antibody (Ab) depends on the preservation of its antigen-binding sites. Our goal was to radiolabel an antibody at several iodine:antibody molar ratios under conditions protecting its combining site and to compare its immunoreactive fraction (IRF) and electrophoretic mobility with those of the same antibody radiolabeled without protection. The data indicate that an antibody radiolabeled while its antigen-binding site is occupied by its antigen had the same IRF, regardless of the number of iodine atoms per antibody molecule. On the other hand, even at an I:Ab ratio of 1:1, the IRF of the same antibody radiolabeled without protection was lower than that of a protected one and decreased with increasing I:Ab ratios. In addition, the iodination of these Ab changes their electrophoretic mobility; however, when the Ab is labeled in the protected state, the degree of change is less. The binding of an antibody to its antigen prior to radiolabeling, therefore, enhances its immunointegrity and prevents major conformational changes as reflected by electrophoresis.

J Nucl Med 1991; 32:116–122

It is generally accepted that the immunointegrity of an antibody depends on the preservation of its antigen-binding sites. The consequence of damaging such sites during radiolabeling is significant, especially since a monoclonal antibody (MAb) population is expected to be uniformly affected by the chemical reactions involved. For example, the presence of a conjugated molecule may alter the conformation of the combining site, especially if the agent is distinctly hydrophobic, possesses multiple charged groups, or causes stearic hindrance of antigen binding (*1*). These effects are also likely to become more pronounced as the degree of radiolabeling increases. Studies have repeatedly demonstrated that having more than two iodine atoms per antibody molecule can seriously interfere with the immunologic function of radiolabeled antibodies and their biologic half-life in the blood stream (*2–7*). In an attempt to protect antigen-binding sites, we have examined an approach in which the antibody is reacted first with its specific antigen and then radiolabeled while maintaining the antigen-antibody complex configuration (protected antibody). We radioiodinated the antibody at various I:Ab molar ratios and compared its immunoreactive fraction and electrophoretic mobility with those of the same antibody radiolabeled at identical I:Ab ratios in the absence of the antigen-antibody complex (unprotected antibody).

Received Jan. 30, 1990; revision accepted Jun. 11, 1990.

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MATERIALS AND METHODS

Antigen-Antibody System

Affinity-purified goat anti-mouse IgG (heavy and light chains) and rabbit anti-mouse IgG (heavy and light chains) at a concentration of 1.8 mg/ml in 0.01 *M* sodium phosphate/0.25 *M* NaCl, pH 7.6, were used as antibodies. Mouse IgG (whole molecule) covalently bound to agarose gel at a concentration of 1 mg protein/ml agarose beads in suspension in 0.01 *M* sodium phosphate/0.25 *M* NaCl, pH 7.6, was used as the antigen. All proteins were purchased from Jackson Immunoresearch Laboratories Inc., West Grove, PA.

Iodination Following the Protection of the Antigen-Binding Sites

All procedures were performed at room temperature. Goat anti-mouse IgG (GAM) (0.5–1 mg) was loaded onto the affinity gel (MIgG covalently attached to agarose beads) pre-equilibrated in 0.03 *M* borate buffer, pH 8.2. The column was washed with the same borate buffer to remove any excess unbound GAM. The GAM-MIgG-gel suspension was then divided into three equal fractions, transferred into glass tubes, and radioiodinated with carrier-added $Na^{125}I$ (37 MBq/mCi; 100 mCi/ml) in the presence of chloramine-T (CT) (CT:Ab, 0.5:1, w/w). Sodium iodide was added first, followed by CT. The mixture was incubated for 1 min, and the reaction was quenched with the reducing agent sodium metabisulfite (6.3 m*M* in 50 m*M* phosphate, pH 7.0). While keeping the protein, CT, and sodium metabisulfite concentrations constant,

the molar I:Ab ratios were varied from 1:1 to 15:1 and 35:1 (Fig. 1).

Following radiolabeling, the gel suspensions were reloaded onto mini-columns (BioRad) and washed with 0.03 *M* borate buffer, pH 8.2. When all unbound radioactivity had been washed off the column, the ^{125}I-GAM was eluted from the affinity gel using the same borate buffer at pH 2.5. These fractions were rapidly brought to pH 8.0 with 1 *M* Tris buffer, pH 8.0, and their radioactive content was determined. The fractions containing the protein peak were pooled and concentrated with a concomitant change of buffer to phosphate-buffered saline (20 m*M* KCl, 136 m*M* NaCl, 8 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1.5 m*M* KH_2PO_4, pH 7.4 [PBS]) using Centricon microconcentrators (Amicon, Danvers, MA). The optical density of the sample was measured at 280 μm and the protein concentration was determined using a standard plot of protein concentration versus optical density. The same protein concentrations were used for direct radiolabeling of the unprotected antibody (vide infra). Three aliquots (10 μl each) were counted in a gamma counter (Packard Instrument Company, Downers Grove, IL) to measure the total amount of activity in each sample and protein-bound activity was assessed by thin-layer chromatography (ITLC SG, Gelman Sciences, Ann Arbor, MI) (vide infra).

Because of the extreme sensitivity of the GAM to certain manipulations, such as the elution step, with subsequent loss of immunoreactivity (unpublished data), we repeated the experiments using rabbit anti-mouse IgG (RAM). RAM was first tested for its sensitivity under low pH conditions and was found able to withstand the affinity chromatography procedure without any measurable loss of immunoreactivity under the experimental conditions. The radiolabeling procedures were then repeated using RAM radioiodinated at an I:Ab ratio of 1:1 following binding to the MIgG-agarose gel. After labeling, the gel suspensions were reloaded onto mini-columns, washed with 0.2 *M* phosphate buffer/0.5 *M* NaCl, pH 7.6, and eluted with 0.2 *M* glycine/0.5 *M* NaCl, pH 2.8, to release the iodinated RAM.

Direct Iodination

GAM or RAM was directly radioiodinated (without protection) (Fig. 1) using the same I:Ab and CT:Ab molar ratios as described above for the protected antibodies. Following iodination, the reaction mixtures were chromatographed on Sephadex G-25 columns that had been pre-equilibrated with 1% bovine serum albumin in PBS. The columns were eluted with PBS, and the void volume fractions (0.5 ml each) containing the protein peak were pooled. The radioactive content of three 10-μl aliquots from the pooled protein was determined as described above. Protein-bound activity was assessed by thin-layer chromatography (TLC, vide infra).

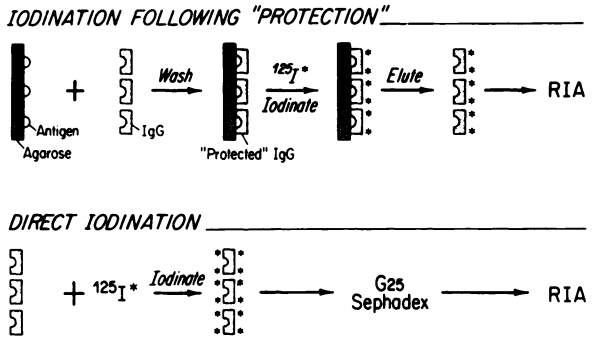

FIGURE 1
Schematic illustration of methods used to iodinate antibody directly or following protection.

Radiolabeling Yield

Aliquots of the radioactive mixtures were loaded near the bottom of 1 cm × 15 cm ITLC SG strips. The strips were placed in a chamber containing 80% methanol as solvent. Once the buffer had migrated 10 cm up to a preset mark, the strips were cut at the 5-cm level and each half was counted in a gamma counter. Each test was done in triplicate. The percent labeling yield was calculated as: counts on bottom half divided by total counts (top + bottom) multiplied by 100.

Immunoreactivity Testing

Each assay was performed on the directly radiolabeled "unprotected" antibodies and on the radiolabeled "protected" antibodies at the respective I:Ab ratios. Testing occurred under conditions of antigen excess. Ninety-six well radioimmunoassay plates were coated with doubling dilutions of the antigen MIgG and incubated overnight at 4°C. The wells were then washed with PBS containing 0.05% surfactant (Calbiochem, La Jolla, CA). Nonspecific binding was assessed by adding an excess of unlabeled GAM or RAM to a series of wells. A constant amount of ^{125}I-GAM or ^{125}I-RAM in 50 μl was then added to all wells, and the plates were incubated for 1 hr at room temperature. Each assay was done in triplicate. Following incubation, the unbound immunoglobulin was removed by washing the plates three times with distilled water. The bound counts were measured by cutting out the individual wells and determining their radioactive content in a gamma counter.

The results were plotted as a double-inverse immunoreactivity plot (total amount of radioactivity applied divided by the activity bound as a function of the inverse of the antigen concentration). The immunoreactive fraction (IRF) was determined by extrapolating this line to the intercept with the ordinate and was expressed as the inverse of the intercept value. The association constant (K_a) was then calculated from the slope and the immunoreactive fraction using the following formula derived from the theoretical analysis of the antigen-antibody binding reaction as described by Lindmo et al. (*8*):

$$\text{slope} = \frac{1}{r \times K_a}$$

where 1/r is the IRF.

Determination of Electrophoretic Mobility

The mobility of the GAM radiolabeled at different I:Ab ratios was determined by agarose gel electrophoresis (Paragon SPE kit: 1.0% agarose; 1.2% barbital buffer, pH 8.6; 0.1% sodium azide) with a Pharmacia flat bed electrophoresis 3000 apparatus (Beckman Instruments, Inc., Fullerton, CA). The procedure was performed as recommended by the manufacturer. Briefly, the protein sample (in a 2-μl volume) was applied to the surface of the gel along a line placed in the middle of the gel. The gels were then fixed on a support and run at 100 V for 25 min. Once focusing was complete, the gels were fixed in an acid-alcohol solution (60% methanol/

40% glacial acetic acid in distilled water) for 3 min, dried, and stained with Coumassie Blue for 3 min. Since the protein concentration was too low to allow visualization of the protein band, the templates were cut parallel to the loading line into 2-mm pieces and each piece was counted in a gamma counter. The results were then plotted as radioactivity versus electrophoretic mobility (mm).

RESULTS

Radiolabeling Yield

The radiolabeling yield as assessed by TLC was >97% for the protected antibodies and >95% for the unprotected antibodies for all I:Ab ratios.

Immunoreactivity

The data for a typical experiment (Fig. 2) indicate that all the protected ^{125}I-GAM samples had the same IRF (8%), i.e., the same intercept with the ordinate, regardless of the number of iodine atoms per antibody molecule. On the other hand, all the antibodies directly radioiodinated in the absence of antigen-antibody complex formation had a lower IRF (5% [1:1], 2% [15:1], 1% [35:1]). While this was expected for the antibodies directly radiolabeled at the higher I:Ab ratios, the antibody radiolabeled at an I:Ab ratio of 1:1 also showed a lower IRF. The affinity constants ($K_a \pm$ s.e.m.) for all the protected antibodies were close (Table 1), varying from $[1.68 \pm 0.13]10^5\ M^{-1}$ at a 1:1 I:Ab ratio to $[1.69 \pm 0.31]10^5\ M^{-1}$ at a 35:1 ratio. The directly radiolabeled antibodies on the other hand had lower K_a's varying from $[3.29 \pm 0.52]10^4\ M^{-1}$ (1:1) to $[6.27 \pm 1.67]10^3\ M^{-1}$ (35:1). This representative experiment exemplifies the overall low IRF observed for all the iodinated GAM samples which is probably due to the extreme sensitivity of this affinity-purified antibody.

When we repeated the experiments using RAM radioiodinated at an I:Ab ratio of 1:1, we again observed that the protected ^{125}I-RAM had a higher IRF. In a characteristic experiment, the IRF for the protected ^{125}I-RAM was 56.8% compared to 26.5% for the directly radioiodinated RAM (Fig. 3). The K_a of the protected RAM was $[7.15 \pm 0.22]10^6\ M^{-1}$ and of the unprotected RAM $[5.92 \pm 0.09]10^5\ M^{-1}$.

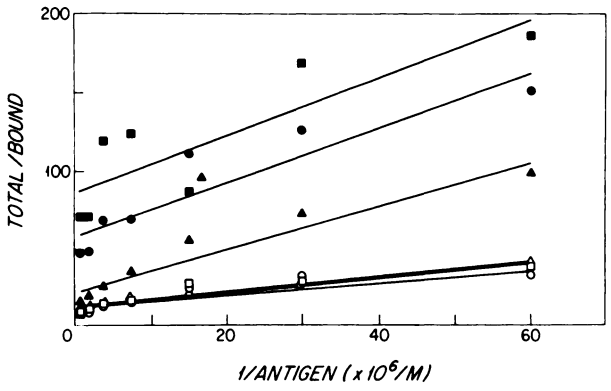

FIGURE 2
Double inverse immunoreactivity plot of directly iodinated goat anti-mouse (I:Ab of 1:1 = ▲, 15:1 = ●, 35:1 = ■) versus iodinated goat anti-mouse following protection (I:Ab of 1:1 = △, 15:1 = ○, 35:1 = □).

Electrophoretic Mobility of Directly Iodinated GAM Versus GAM Iodinated Following Protection

When the electrophoretic mobility of the ^{125}I-GAM radiolabeled at three I:Ab ratios (1:1, 15:1, 35:1) either directly or following protection of the antigen-binding sites was compared to that of the native unlabeled GAM, we observed that the protected antibodies moved toward the cathode (Fig. 4) in a manner similar to native unlabeled GAM. The extent of electrophoretic mobility toward the cathode was a function of the number of iodine atoms per antibody molecule: the higher the I:Ab ratio, the stronger the change in the mobility of the protein. The directly radiolabeled GAM at a 1:1 I:Ab ratio remained at the origin while moving toward the anode at higher I:Ab ratios (15:1, 35:1), the extent of the mobility depending again on the number of iodine atoms per antibody molecule. The binding of the antibody to its antigen prior to radiolabeling appears, therefore, to induce less of a change in the electrophoretic mobility of the antibody (even at high I:Ab ratios) compared to the same antibody radiolabeled at identical ratios when unprotected.

DISCUSSION

The binding of an antibody to a protein antigen is a noncovalent and reversible reaction that shares many features in common with the binding to haptens (*9*). Therefore, regardless of the size of the antigen, similar changes are observed among antibody molecules when they bind to their specific antigen. This interaction induces functionally important conformational changes that improve the stereochemical complementarity between the interacting molecules (*10*), contribute to the formation of a stable complex in which the sensitive portion of the antibody molecule is in close contact with the antigen surface and buried into the complex, and result in a tightly packed interface which mainly excludes solvent (*11*). Cathou et al. (*12,13*) demonstrated that the susceptibility of the antibody molecules to unfolding when exposed to a dissociating agent such as guanidine hydrochloride was greatly reduced after antigen-antibody complex formation. Moreover, their antigen-binding activity was still intact at high concentrations of the dissociating agent, while, conversely, the antibody without its antigen readily unfolded at lower ones.

X-ray crystallographic studies of the structure of antigens, antibodies and antigen-antibody complexes have defined antigen-antibody reactions at the molecular level (*14*) and have shown that maintaining the structure of the antigen-binding sites is important. Any

TABLE 1
Association Constants and Immunoreactive Fractions for GAM* and RAM†

	GAM		RAM	
I:Ab‡	Protected§	Direct¶	Protected	Direct
	Association constants (K_a)			
1:1	$(1.68 \pm 0.13)10^5\ M^{-1}$**	$(3.29 \pm 0.52)10^4\ M^{-1}$	$(7.15 \pm 0.22)10^6\ M^{-1}$	$(5.92 \pm 0.09)10^5\ M^{-1}$
15:1	$(1.97 \pm 0.56)10^5\ M^{-1}$	$(9.74 \pm 1.90)10^3\ M^{-1}$		
35:1	$(1.69 \pm 0.31)10^5\ M^{-1}$	$(6.27 \pm 1.67)10^3\ M^{-1}$		
	Immunoreactive fractions (IRF)			
1:1	8%	5%	57%	26%
15:1	8%	2%		
35:1	8%	2%		

* GAM = Goat anti-mouse IgG.
† RAM = Rabbit anti-mouse IgG.
‡ I:Ab = iodine to antibody molar ratio.
§ Protected = radiolabeled after antigen-antibody complexation.
¶ Direct = directly radiolabeled.
** Mean ± s.e.m.

modification (substitution, deletion, or insertion) in the amino acid sequence of the hypervariable region is known either to alter the depth of the cleft (*15*) or to allow certain local side-chain rearrangements that result in the positioning of new side chains in the combining site and a subsequent reduction in the binding strength of the antibody (*10*). In addition, circular dichroism and fluorescence polarization measurements (*16,17*) as well as kinetic studies (*18,19*) have indicated that the folding pattern of the polypeptide chain that accompanies the connection between an antigen and an antibody is different from that of the same immunoglobulin in the absence of its antigen. Unfortunately, the crystallographic data on a given antibody in both free and bound forms are scarce and only a few three-dimensional structures of antigen-antibody complexes have been published (*9,11,20*).

All these observations suggest that the three-dimensional conformation or quaternary structure of an antibody bound to its antigen tends to protect its sensitive portion by rearrangement so that most of the interface or at least the amino acids most involved in antigen-binding are buried in the protein interior and, therefore, less accessible. This may be of significance when one considers radiolabeling small antibody fragments, since the probability of having the conjugate or radiolabel located in the sensitive portion of the antibody would be expected to be much higher.

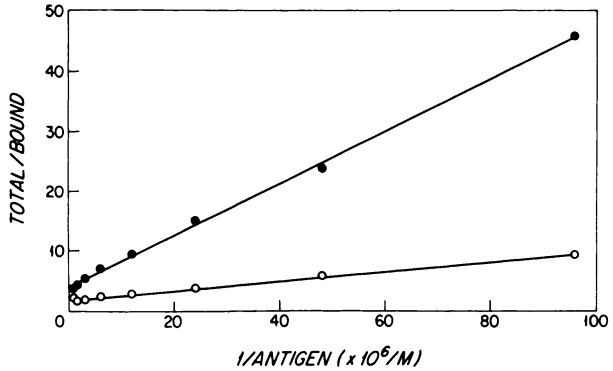

FIGURE 3
Double inverse immunoreactivity plot of directly iodinated rabbit anti-mouse (●) versus iodinated rabbit anti-mouse following protection (○) at I:Ab ratio of 1:1.

The susceptibility to denaturation during radiolabeling and the conditions for successful radiolabeling differ dramatically for various MAb (*1,2,7,21-22*). Even a very slight denaturation of an antibody during labeling may alter its pharmacokinetics in vivo (*4,23*). The antibody sites targeted during a radiolabeling procedure include (*24*) tyrosine residues (*1,25*), oligosaccharide moieties (*26*), ε-amino groups of lysine (*24,27*), β- or γ-carboxyl groups of aspartic and glutamic acids (*28*), and thiol groups generated by reduction of cystine (*29*). Each of these groups however, does not seem to be equally available for conjugation (*24*). Coupling of only a few molecules to the amino groups of certain monoclonal antibodies has been shown to decrease antigen-binding (*24*), suggesting that amino groups important for such binding are more reactive than others and undergo covalent coupling first (*2,24*). Endo (*24*) has recently postulated that it should be possible to modify these reactive amino groups preferentially with a reversible amino-group-blocking reagent (2,3-dimethylmaleic anhydride [DMA]) and has demonstrated that a methotrexate-MAb conjugate prepared by the DMA method retained full antigen-binding capacity compared with that of the same conjugate prepared by a conventional method.

In our approach, we directed the radiolabel away from the combining site of the immunoglobulin by

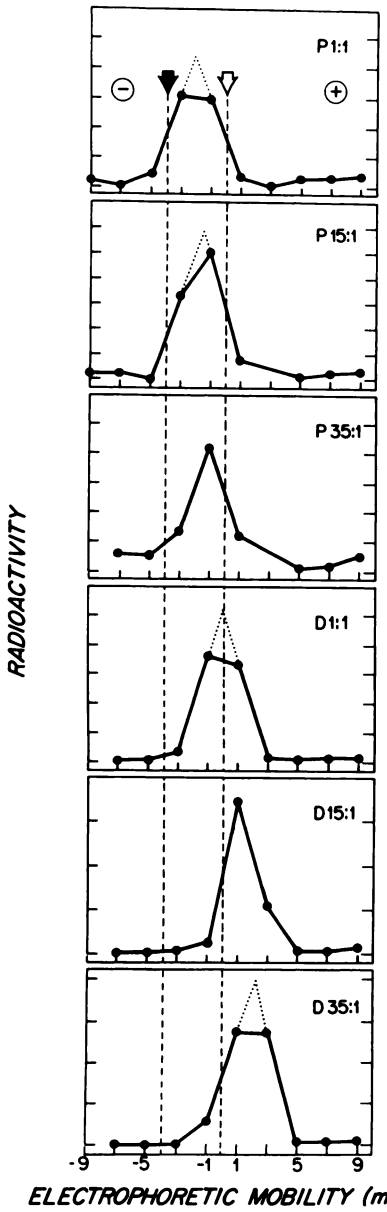

FIGURE 4
Electrophoretic mobility (EM) of GAM radioiodinated at three I:Ab ratios (1:1, 15:1, 35:1) either directly (D) or following protection (P). Closed arrow indicates EM of unlabeled GAM and open arrow point of origin (sample loading zone). When definite peak of activity was not apparent, slopes of activity curve were extrapolated (dotted lines) to indicate approximate EM of protein. Negative numbers indicate that protein moved toward cathode (−), while positive numbers indicate protein moved toward anode (+).

radiolabeling the antibody when it was bound to its antigen. Our hypothesis was that by having the cleft, which consists of the folded hypervariable regions, occupied by the antigen, we might be able to maintain the integrity of those regions directly in contact with the antigen. To test this hypothesis, we deliberately chose to radiolabel antibodies by a direct radioiodination method at high I:Ab ratios knowing that, under these conditions, most antibodies are likely to be severely damaged. We reasoned that if the protection of the antigen-binding sites resulted in any improvement in immunoreactivity under these extremely harsh conditions, it would be worthwhile exploring this technique. Our results indicate that the binding of the antibody to its antigen prior to radiolabeling preserved its immunointegrity, regardless of the number of iodine atoms per antibody molecule. Not only was the IRF of the protected antibodies higher than that of the same antibody directly radiolabeled even at the conventional I:Ab ratio of 1:1, but all the protected antibodies had an identical IRF, independent of the number of iodine atoms per molecule. Similarly, the association constants for all the protected antibodies were nearly identical, while those for the directly radiolabeled antibodies were significantly lower, decreasing as a function of the number of iodine atoms per antibody molecule. These observations suggest that the three-dimensional rearrangement that follows the interaction of these antibodies and their antigens may help preserve the combining site and direct the label onto areas of the polypeptide chain that are not essential to the antibody function. A similar conclusion was drawn by Pressman and Sternberger in 1951 (*30*) in a perceptive article in which these authors radioiodinated two different rabbit antisera in the presence or absence of their specific hapten at various I:Ab ratios. Day et al. (*31*) used a similar approach to radioiodinate rabbit anti-porcine fibrinogen serum in the presence or absence of fibrin and also demonstrated that the preadsorption of the serum to fibrin preserved the reactivity of the antibodies.

Our electrophoretic studies also show that the protected antibodies had an overall mobility similar to that of the native protein even at high I:Ab ratios. Here again, the quarternary structure of the antibody bound to its antigen may have reduced access of the oxidant (CT) to certain parts of the molecule resulting in little change in the overall charge of the protein. This preservation of the original charge may also play a favorable role in the attraction between an antibody and its antigen since electrostatic forces and the hydrogen bonding capacity of the charged molecule are known to play a role in antigen-antibody interaction. Indeed, such attraction may increase the rate of antibody-antigen complex formation by facilitated diffusion and stabilize the initial complex (*10*). It should be noted, however, that while the electrophoretic mobility of ^{125}I-GAM was dependent on the number of iodine atoms per antibody molecule, this dependency was not observed with another iodinated antibody (^{125}I-MIgG) directly radiolabeled at identical I:Ab ratios. In this case, we observed a complete reversal of the mobility of the protein which was independent of the number of iodine atoms per antibody molecule, i.e., all the directly

radiolabeled proteins moved to the same extent toward the anode (unpublished results). Therefore, while the overall modification of the mobility of these two antibodies (GAM and MIgG) following direct radiolabeling is similar, i.e., increase in movement toward the anode, the extent of the change may vary among different proteins, reflecting differences in sensitivity with regard to conformational or other changes secondary to the radiolabeling procedure.

Although some of the principles and approaches of the present work were defined as early as 1951, it is surprising that they have not been widely applied to monoclonal antibody technology despite important advances in immunochemistry and biological techniques. Many tumor-associated antigens have been identified, isolated (*32,33*), and immobilized onto various supports, e.g., beads or plates, for the purpose of radioimmunoassay. However, even if the specific antigen is difficult to isolate or attach onto a matrix, protection of the antigen-binding sites might still be achieved through the use of antibodies directed against the hypervariable regions of the MAb, e.g., anti-idiotypic antibodies. Among these antibodies, the anti-paratope antibodies mimic antigens and, therefore, complement an internal image of the binding site (*34*). Since these antibodies would bind in the same area as does the antigen, they should occupy the combining region and prevent access of other atoms or molecules. In addition, improvements in elution buffer have been made, and proteins can now be eluted from affinity gels at pH 6.5.

In conclusion, the binding of an antibody to its antigen during the radiolabeling procedure results in greater immunointegrity compared with the same antibody directly radiolabeled without protection. Existing MAb technology could apply this technique in the conjugation and/or radiolabeling procedures already in use. Further investigations into the three-dimensional structure of antigen-MAb complexes (whole molecule or fragments) following conjugation and/or radiolabeling may help elucidate the conformational and functional changes imposed upon the MAb molecule. These findings could then help in the development of methods that would result in the production of immunointact radiolabeled MAb for diagnosis and therapy.

ACKNOWLEDGMENTS

Presented in part at the Fourth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, CA, March-April 1989 and at the 36th Annual Meeting of the Society of Nuclear Medicine, St. Louis, MO, June 1989. Financial support was provided by DOE Grant DE-FG02-86ER60460.

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EDITORIAL

The Advantage of Protecting the Antigen-Binding Site During Antibody Labeling

Radioimmunotherapy and radioimmunoscintigraphy have emerged in recent years as fields of active research and development in nuclear medicine. There are large numbers of antibodies currently being evaluated in clinical trials for a variety of diseases. With several years of collected clinical experience, one limitation has consistently emerged; antibodies have low binding to target sites in vivo. Most investigators observe radioactivity concentrations in the range of 10^{-4}% of the injected dose per gram. Because of this low target binding, chemists are exploring methods for increasing antibody deposition in target sites, to improve the clinical utility of these materials. Since reduced immunoreactivity in vivo can result from effects of radiolabeling, approaches to minimize these detrimental effects may increase tumor binding and retention of radiolabeled antibody at the target site.

The article by Van den Abbeele et al. (*1*) presents a comprehensive study of the factors that can contribute to reduced immunoreactivity of radiolabeled antibody, as well as a unique approach to improving the quality of these new radiopharmaceuticals. After a clear presentation of the importance of conformational changes in the antibody tertiary structure associated with antigen binding, and a discussion of the effects of added radioiodine atoms on these critical conformational changes, the authors hypothesized that protection of the critical binding site during radiolabeling would improve the immunointegrity of the final product. To test this hypothesis, they designed an experiment where an antigen (a murine antibody) was adsorbed onto a stationary gel. The antibody to be labeled (goat or rabbit anti-mouse antibody) was allowed to bind to the stabilized antigen; thereby, protecting the critical binding site of the antibody from addition of radioiodine atoms. This was followed by standard radioiodination with chloramine-T. Following radioiodination, the labeled antibody was eluted from the antigen and critically assayed in a variety of ways. Antibodies labeled by this method were compared to those labeled without protection of the antigen-binding site. All antibodies were labeled at a series of iodine-to-antibody molar ratios that were purposely high to stress any adverse effects of increased substitution of radioiodine atoms per antibody.

Radiochemical purity was determined by thin-layer chromatography. Immunoreactivity assessed by Scatchard analysis was determined by the use of a cell binding assay at antigen excess, and antibody net charge was evaluated by isoelectric focusing techniques. Radiochemical purity for each group of antibody

Received Sept. 6, 1990; accepted Sept. 6, 1990.
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