Pharmaceutical Research, Vol. 9, No. 11, 1992

Review

Monoclonal Antibodies in Hapten Immunoassays

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This review deals with the potency of monoclonal antibodies (MAbs) to haptens in immunoassays. Specificity and affinity of MAbs to haptens are the major determinants to be considered. Specificity of MAbs depends on the selection of the hapten coupling site to the carrier protein and the antigen used for the screening of MAbs. Nevertheless, cross-reactivity can occur with compounds related to the hapten. This polyspecificity may be circumvented with the use of many MAbs, as has been demonstrated for MAbs to cyclosporine. Affinity of MAbs to haptens is often lower than that of corresponding polyclonal antibodies (PAbs), thereby limiting assay sensitivity. Low affinity is more frequently observed with low molecular weight (100–300) haptens than with larger haptens, such as digoxin or cyclosporine. Affinity enhancement by increasing resemblance to the immunogen can be effective in resolving the lack of sensitivity. With suitable selection strategies, MAbs exhibit real advantages over classical PAbs to haptens because large amounts of worldwide standardized reagents can be prepared.

KEY WORDS: monoclonal antibody; hapten; specificity; affinity.

INTRODUCTION

Since the first description by Köhler and Milstein in 1975 (1), monoclonal antibodies (MAbs) have played an increasingly important role in detecting low molecular weight compounds or haptens. However, they have not replaced polyclonal antibodies (PAbs) in immunodiagnostics because MAb specificity and affinity may be insufficient.

This review deals with the advantages and limitations of MAbs for immunoassay with small haptens. For the selection of suitable MAbs, two major problems can arise. The affinity of MAbs can be too low (often lower than with PAbs) and lead to an insufficient detection limit, or low specificity of MAbs to small haptens results in cross-reactivity with hapten analogues (often broader than that of PAbs). Thus, the selection of available MAbs has to be adapted according to the analytical aim of the immunoassay.

SPECIFICITY OF MAbs TO HAPTENS

As MAbs are issued from a unique B-lymphocyte clone, one often assumes that all MAbs are immunologically monospecific. In reality, many MAbs to haptens are polyspecific. Two types of polyspecificity can occur. Controlled polyspecificity is represented by the cross-reactivity toward compounds with the same chemical skeleton with varying functional groups (methyl, hydroxyl. . . .) such as drug metabolites. This type of polyspecificity is easily verified by standardized cross-reactivity studies. In contrast, uncontrolled polyspecificity involves the cross-reactivity of MAbs with compounds different from the native compound, such as another drug(s), peptide(s), or protein(s). The latter compounds are not always identified and often do not present strong structural or chemical analogies with the hapten, thus precluding cross-reactivity studies.

Controlled MAb Polyspecificity

Although it is difficult to predict epitopes targeted by the humoral response, two determinants of antibody specificity have to be considered prior to the production of MAbs: the attachment site of the hapten to the carrier protein and the screening procedure for individual MAbs.

Influence of the Hapten/Carrier Protein Coupling Site. The choice of the hapten coupling site is a major determinant of antibody specificity. Thus, before the preparation of MAbs, the metabolic pathway of the hapten should be clearly established. If the epitope is metabolized, the product cannot interact with the antibody. In contrast, if biotransformations occur outside the epitopic zone, all the molecules can be recognized by the antibody. For example, the neutral undecapeptide cyclosporine is extensively metabolized to at least 20 metabolites (2). The sites of biotransformation involve amino acid residues 1, 3, 4, 6, and 9, but the metabolism does not modify the rigid skeleton. It seems that *in vivo* the major metabolites do not have pharmacological activity, but some may be toxic. PAbs are too polyspecific and HPLC is too time-consuming for monitoring transplant patients. To resolve these problems, MAbs to cyclosporine have been developed. Amino acids 2 and 8 were selected as metabolically inert coupling sites to the carrier

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0724-8741/92/1100-1375$06.50/0

protein (3). As cyclosporine possesses no functional groups suitable for coupling to a carrier protein, two synthetic analogue peptides, *d*-Lys_8-cyclosporine and Thr_2-cyclosporine, were used as drug–carrier complexes. Cross-reactivities of the resultant MAbs with the major human metabolites showed that MAbs to cyclosporine reacted with a mosaic of overlapping epitopes on the antigen principally opposite to the coupling site (3). Two MAbs were selected, one strictly monospecific against the native cyclosporine, excluding interference from all metabolites, and a second which recognized native cyclosporine and most of its major metabolites. Finally, these specificity data were verified with plasma from cyclosporine-treated patients by comparing with HPLC and immunoassay results using MAbs and PAbs (4). Cyclosporine concentrations obtained with the monospecific MAbs were compatible with those of HPLC. In contrast, polyspecific MAbs and PAbs gave higher cyclosporine concentrations. Thus, despite an identical immunogen preparation, monospecific or polyspecific MAbs to cyclosporine could be developed.

The pharmacological activity of most racemic drugs such as β-blockers is stereoselective, and hence, the assay must be stereoselective to investigate pharmacokinetics of the active isomer. When the immunoconjugate used for the immunization was prepared with the racemic form of propranolol (5), two populations of MAbs were theoretically produced, directed against each enantiomer. In fact, the two selected MAbs to propranolol bound both optical isomers and were strictly not stereoselective. To raise stereospecificity of MAbs, the purified enantiomer can be coupled to the carrier protein at the site opposite to the asymetric carbon. This strategy has been applied to the preparation of MAbs to *l*-alprenolol (6) and abcisic acid (7). However, stereospecificity is not absolute, and the opposite enantiomer was found to be weakly bound by the MAb.

Attachment site of the carrier protein may be unrelated to the polyspecificity phenomenon. In our laboratory, PAbs and a MAb to the antigout drug colchicine were developed using the same immunogen, 4-hydroxymethyl colchicine hemisuccinate coupled to bovine serum albumin (BSA) (8). Specificity studies of these two antibodies showed surprisingly that PAbs exhibited a more restrictive specificity than the MAb. Thus, the MAb recognized not only the native colchicine but also 2- and 3-demethylated colchicine metabolites (Table I). These cross-reactivity studies indicated that the recognition of colchicine by its MAb was restricted to rings B and C of colchicine, allowing cross-reactivity with all metabolites arising from chemical modifications on ring A. In contrast, for the PAb to colchicine, recognition involved determinants of the three rings, limiting cross-reactivity to metabolites. Analysis of colchicine immunoreactivity in serum was considerably higher when using the MAb than with the PAb, which precludes its use in pharmacokinetic studies (Fig. 1), and it illustrates the risk of MAb polyspecificity. Thus, when Quesniaux *et al.* (3) developed MAbs to cyclosporine, they selected only 2 MAbs for therapeutic drug monitoring of the 180 initially prepared. The wider the MAb panel, the easier the selection of a defined specificity for the desired analytical profile.

Influence of the Antigen Used for the MAb Screening-Procedure. Among the different steps of MAb preparation, screening is essential before large-scale production. To obtain suitable MAb specificity, the same chemical compound should be used both as the immunogen and as the labeled antigen in the screening of antibodies. When two distinct compounds have the same chemical skeleton, the risk of selecting MAbs with polyspecificity is increased. Thus, De Blas *et al.* (9) developed MAbs to benzodiazepines using clonazepam as the drug–protein complex for immunization and ^{3}H-flunitrazepam as the radioligand for the screening. As a result, the MAbs recognized flunitrazepam (K_a = $6.8 \cdot 10^{10}\ M^{-1}$) better than clonazepam ($K_a = 3 \cdot 10^7\ M^{-1}$). For thus, the selected MAbs presented a similar affinity for other benzodiazepines, such as diazepam and Ro 5-6896. For the screening of MAbs, when there is no homologous labeled drug for the specificity study of the antibody, the enzyme-linked immunosorbent assay (ELISA) procedure can be used with the hapten coupled to the same site as that used for the protein carrier attachment but with a different carrier protein, so as to avoid polyspecificity phenomena with the carrier protein.

Table I. Comparison of Antibodies to Colchicine Raised Against the Same Hapten[a]

Compounds	% of cross-reactivity	
	PAb	MAb
Colchicine	100	100
2-Demethylcolchicine	9	100
3-Demethylcolchicine	15	100
N-Desacetylcolchicine	1	2
Colchiceine	0.05	0.06

[a] Cross-reactivity is expressed as the ratio of the colchicine concentration to the cross-reacting substance concentration at 50% inhibition of maximum binding.

Uncontrolled MAb Polyspecificity

Sometimes natural or synthetic compounds without a skeleton similar to the immunogen are able to bind to the prepared MAbs (10). This polyspecificity was first reported

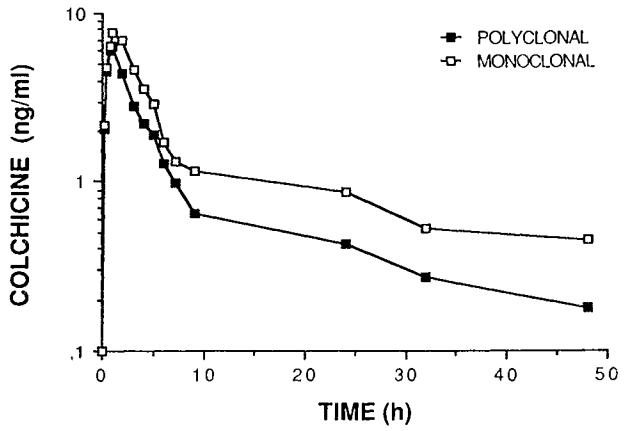


Fig. 1. Pharmacokinetics of oral colchicine (1 mg) measured in plasma by radioimmunoassay using specific PAbs and a polyspecific MAb.

by Richards *et al.* (11), who showed that a homogeneous antibody population (myeloma Ig A) was able to bind two small and structurally unrelated molecules: dinitrophenyl-lysine and menadione. These two compounds competed for the paratope at distinct but overlapping sites with low association constants ($2 \cdot 10^4\ M^{-1}$ for dinitrophenyl-lysine and $1 \cdot 10^5\ M^{-1}$ for menadione). Later, Innan and Barnett (12) studied the cross-reactivity of a MAb against dinitrophenyl with 85 diverse compounds; three compounds were able to bind to the same MAb with association constants of similar magnitude (K_a, about $10^5\ M^{-1}$) but lower than with the native hapten ($K_a = 10^7\ M^{-1}$). These interfering compounds have no structural homology with the immunogen 2,4-dinitrophenyl-aminoalkyl, and the electrical charges of the compounds cannot be implicated in the polyspecificity phenomenon. These results show that the interacting compounds can bind to the paratope of the MAb at sites other than those interacting with the original hapten. Uncontrolled polyspecificity is often discovered at a late stage during the development of the immunoassay, as has been observed also for MAbs to metamphetamine which bind ranitidine (13). One of the difficulties in digoxin therapeutic monitoring is the existence of digoxin-like immunoreactive substances (DLIS) (14). As DLIS structures have not been identified, the interference of these endogenous compounds illustrates the term uncontrolled polyspecificity. A cross-reactivity study with seven MAbs to digoxin showed that two MAbs cross-reacted with DLIS present in cord blood plasma (15). One of these MAbs presented a high K_a for digoxin ($1.2 \cdot 10^{12}\ M^{-1}$) but exhibited a high cross-reactivity with DLIS. Hence, a high affinity for the analyte does not exclude interference by unknown compounds.

AFFINITY OF MAbs TO HAPTENS

The limit of detection is the other major criterion which decides the success of the immunoassay. As the association constant is inversely related to the limit of detection, the preparation of high-affinity MAbs to haptens increases the chances of obtaining a satisfactory detection limit. Other contingent factors, such as duration of assay incubation and experimental errors, also play a limiting role (16). Thus, it would be theoretically possible to detect compounds in the nanomolar concentration range by using MAbs with K_a values in the $10^9\ M^{-1}$ range (17). Nevertheless, the affinities of MAbs are usually lower than those observed for PAbs. Of 62 published MAbs to digoxin (18–23), only 19 MAbs had higher affinities than those described with purified polyclonal sheep antibodies to digoxin ($K_a = 1.4 \cdot 10^{10}\ M^{-1}$) (24). Another example was the apparent K_a of a MAb to nortriptyline ($2.9 \cdot 10^7\ M^{-1}$) which was lower than that for the corresponding PAbs ($1.1 \cdot 10^9\ M^{-1}$) (25). However, for these drugs the development of immunoassays with MAbs was not limited by these weaker K_a values because their therapeutic ranges was in the $10^{-6}\ M$ range. In contrast, for a drug with a therapeutic range of the order of nanomolar, a lower K_a could be a limiting factor for immunoassay performance. Limited MAbs affinity to haptens can be related to the hapten molecular weight or to the area of interaction between epitopes and antibody binding sites. Recently, the area of the interaction binding between 2-phenyloxazole (MW 162) and its specific MAb was evaluated at 400 $Å^2$ (26), while classical binding areas were reported at about 700 $Å^2$ (27). Table II shows that for haptens with molecular weights in the range of 100–300, the K_a values of MAbs ranged from 10^4 to $10^8\ M^{-1}$ and are lower than with PAbs to similar haptens. K_a values approach those of PAbs only with higher molecular weight compounds, e.g., digoxin and cyclosporine, which are able to fill the paratope. Despite their heterogeneity, PAbs are therefore often preferred to MAbs for immunoassays. For enhancement of the MAb affinity, two strategies can be proposed: increasing resemblance to the immunogen or mixing several MAbs.

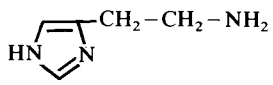
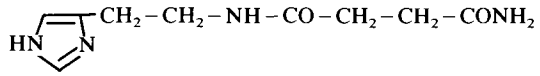
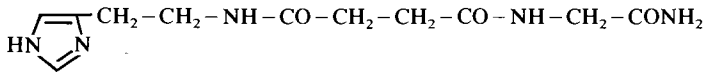
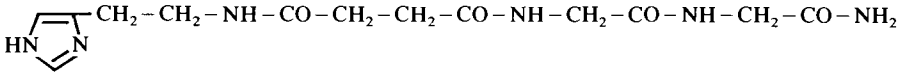
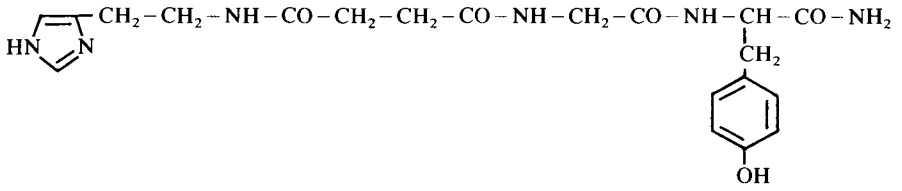
Affinity Enhancement by Increasing Resemblance to the Immunogen. Morel *et al.* (28) have recently succeeded in enhancing the affinity of histamine (a small hapten; MW 111, with a K_a of $10^4\ M^{-1}$) when histamine is directly coupled to the carrier. By using derivatives including the hapten plus a chemical entity resembling an immunogenic conjugate such as histamine succinyl glycyl tyrosinamide, the authors achieved a K_a of $10^{11}\ M^{-1}$. This study shows that the more the chemical derivatized to histamine resembles the immunogen, the more the K_a of MAbs to histamine is enhanced (Table III). This enhancement is due to a better fit between the hapten and the MAb paratope, i.e., the size and the charge of the linker added. Thus, this method requires chemical pretreatment of the biological sample to transform the analyte into the immunogen structure by a nonspecific chemical derivatization. This strategy allows MAb affinity to be improved by a factor of 10^5, and plasma histamine levels can be easily measured. This approach has also been applied to other haptens such as cAMP (29), serotonin (30), and linolenic acid (31).

Affinity Enhancement by Mixing Several MAbs. Mixing MAbs directed against different epitopes on the antigen may result in a marked enhancement in the avidity of the antibody mixture for the antigen, though the intrinsic affinities of each antigen–antibody combining site have not changed. This increased binding is produced by the formation of multimolecular complexes (32). This effect has been shown using pairs of MAbs against HLA antigen (33) and human chorionic gonadotropin (34). Thus, when two MAbs to human chorionic gonadotropin were mixed, the average affinity was 10 times greater than the individual affinity of the two MAbs. The two MAbs chosen by Moyle *et al.* (34) recognized two different epitopes of the protein leading to a positive cooperative effect. Similarly, a 20-fold increase in the sensitivity of an enzyme immunoassay for the human granulocyte–macrophage colony-stimulating factor was observed by using cocktails of three MAbs with different epitope specificities (35). However, in the case of a small hapten, dinitro-

Table II. Association Constant as a Function of the Hapten Molecular Weight

Hapten	MW	$K_a\ (M^{-1})$	Ref. no.
Histamine	111	10^3–10^4	28
Nortriptyline	263	10^7–10^8	25
Digoxin	780	10^9–10^{12}	20
Cyclosporine	1202	10^{10}–10^{11}	4

Table III. Enhancement of the Affinity Constant by Increasing the Resemblance to Immunogen (28)

Name (formula)	K_a (M^{-1})
Histamine 	$1.2 \cdot 10^4$
Histamine-succinyl-amide 	$7 \cdot 10^6$
Histamine-succinyl-glycynamide 	$9.1 \cdot 10^9$
Histamine-succinyl-glycyl-glycynamide 	$2.6 \cdot 10^9$
Histamine-succinyl-glycyl-tyrosinamide 	$1 \cdot 10^{11}$

phenyl, the equimolar mixing of two antidinitrophenyl MAbs with the same affinity ($K_a = 3 \cdot 10^5\ M^{-1}$) did not enhance the average affinity constant (36). This result is expected because of competition between the two MAbs for the same antigen epitope. It seems plausible that the small size of hapten precludes the formation of higher-order complexes and that synergistic effects observed with mixtures of MAbs to macromolecules cannot be observed in hapten immunoassays.

CONCLUSION

MAbs have several advantages over classical PAbs to haptens. Worldwide standardization of analytical measurements now exists and these reagents have well-defined specificities and affinities. Moreover, specificity and affinity remain stable over a long period and MAbs can be produced in large amounts. However, there are disadvantages such as possible low affinity and polyspecificity. This review shows that the theoretical immunological monospecificty of MAbs does not always confer an advantage over PAbs. If the epitopic zone is located in a restrictive zone of the hapten, cross-reactivity may occur with compounds modified out of the epitope structure. The only way to overcome this polyspecificity is to develop a large number of MAbs, which allows selection of a MAb with the appropriate analytical specificity. In the same way, development of a large number of MAbs can help in the selection of a MAb with the required lower limit of detection. These problems may be major for immunoassays to haptens when specificity and sensitivity criteria are important depending the analytical aim. Despite these limitations in their use, MAbs offer a wide range of analytical application. Bioengineering of MAbs with the aim of improving the binding activity could result in MAbs becoming the antibody of choice in hapten immunoassays in the future.

ACKNOWLEDGMENT

The authors wish to thank Professor D. D. Breimer for suggestions and critical discussions.

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