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Lack of specificity of commercially available antisera against muscarinergic and adrenergic receptors

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Abstract Commercially available antisera against five subtypes of muscarinic receptors and nine subtypes of adrenoceptors showed highly distinct immunohistochemical staining patterns in rat ureter and stomach. However, using the M_{1-4} muscarinic receptor subtypes and α_{2B} -, β_2 -, and β_3 -adrenoceptors as examples, Western blots with membranes prepared from cell lines stably expressing various subtypes of muscarinic receptors or adrenoceptors revealed that each of the antisera recognized a set of proteins that differed between the cell lines used but lacked specificity for the claimed target receptor. We propose that receptor antibodies need better validation before they can reliably be used.

Keywords Antibody selectivity · Antibody specificity · Immunohistochemistry · Western blotting

Abbreviations

MR muscarinic receptor
AR adrenoceptor

Introduction

The use of antisera for immunohistochemical or other applications is based on the assumption that they exhibit high specificity for their target. Many commercially available antibodies are stated to be “affinity”-purified, but it is doubtful whether this purification procedure actually improves their specificity. Among the many criteria to evaluate specificity, a claim for selectivity of commercially available antisera is most often based upon a distinct immunohistochemical staining pattern, a single band of the expected size on Western blots, and/or the disappearance of staining after pre-absorption of the antiserum with purified epitope (cf. Petrusz et al. 1976 *versus* Swaab et al. 1977). However, all of these are soft criteria with justifiable exceptions. More meaningful “hard” specificity criteria include a staining pattern that is identical to that of an antibody raised against a different epitope on the same protein (Fischer et al. 2003) or the absence of staining in tissues of animals genetically deficient for the antigen (Swaab et al. 1977; Holmseth et al. 2006; Pradidarcheep et al. 2008). For example, we have recently reported that commercially available antisera against muscarinic receptor subtypes fulfilling at least one of the “soft” criteria lack specificity when tested in mice genetically deficient for the target receptor (Pradidarcheep et al. 2008). Therefore, additional validation of specificity of antibodies is often required.

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While knockout animals are one of the most elegant ways to demonstrate specificity of a given antibody, their routine use for validation has several limitations. Firstly, knockout animals are not available, at least not for some researchers wishing to validate their antisera, for all target proteins. Secondly, an antibody for a given species of interest may not necessarily cross-react with its murine homolog. Finally, it is conceivable that a given antiserum may not be sufficiently specific for use, e.g., in immunohistochemistry but may nevertheless be suitable in Western blotting because band size can be used as an additional criterion to identify the protein of interest. An example of this is commercially available antibodies against G_s proteins which cross-react with smoothelin (Gsell et al. 2000); while this limits their use in immunohistochemistry, such antisera have nevertheless been helpful in studying regulation of G_s proteins by Western blots in many laboratories.

Therefore, we have tested whether genetically modified cell lines that stably express a receptor subtype of interest or related subtypes could be an attractive alternative for the use of knockout animals. Because all antisera against muscarinic receptors (MR) and adrenoceptors (AR) that were investigated failed this test and because this finding appears the rule rather than the exception, we propose to qualify antisera that meet the “hard” specificity criteria as “validated.”

Materials and methods

Antibodies The antisera that were used are described in Table 1. All antibodies were designated affinity-purified preparations by the respective suppliers.

Animals Wistar rats, 4–5 weeks old, were euthanized by instant decapitation under an O₂/CO₂ daze, in agreement with Dutch guidelines for experimental animals.

Immunohistochemical staining The organs of the lower urinary and gastrointestinal tract were fixed overnight at 4°C by immersion in an ice-cold mixture of methanol–acetone–water (2:2:1; v/v). After dehydration and embedding, serial sections (7 μm) were mounted on poly-L-lysine-coated slides (for details, see Pradidarcheep et al. 2008). Antibody concentrations and staining times were chosen to assure a linear relation between antibody binding and staining intensity (van Straaten et al. 2006). Control sections, in which the primary antibody was omitted, were always included in the assays.

Cell lines expressing adrenergic or muscarinic receptors Cell lines stably transfected with the human α_{1A}-, α_{1B}-, or α_{1D}-AR (Rat-1 fibroblasts) or α_{2A}-, α_{2B}-, or α_{2C}-AR (HEK-293 cells; Krege et al. 2000), β₁-, β₂-, or β₃-AR (Chinese hamster ovary (CHO) cells; Niclauß et al. 2006), or M₁-, M₂-, M₃-, or M₄-MR (HEK-293 cells; Schmidt et al. 1995) were maintained as previously described. All cell lines had been tested for the expression of the transfected receptor subtype based on cell signaling responses or radioligand binding.

Western blotting The preparation of whole-cell protein extracts in radioimmunoprecipitation assay buffer containing protease inhibitors, their separation on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and blotting to poly-

Table 1 Antibodies used in this study

Antigen	Host species	Dilution WB	Dilution IHC	Code	Supplier	Blocking serum
MR1	Rabbit	1:900	1:300	AMR-001	Alomone Labs	Goat or fetal calf
MR2	Rabbit	1:600	1:250	AMR-002	Alomone Labs	Goat or fetal calf
MR3	Rabbit	1:750	1:500	AS-3741S	RnD	Goat or fetal calf
MR4	Mouse	1:300	1:50	MAB1576	Chemicon	Fetal calf
MR5	Rabbit	1:1,000	1:1,000	AS-3781S	RnD	Goat or fetal calf
α _{1A} AR	Goat	1:400	1:50	sc-1477	Santa Cruz	Rabbit or fetal calf
α _{1B} AR	Goat	1:400	1:50	sc-1476	Santa Cruz	Rabbit or fetal calf
α _{1D} AR	Rabbit	1:400	1:50	sc-10721	Santa Cruz	Goat or fetal calf
α _{2A} AR	Goat	1:400	1:100	sc-1478	Santa Cruz	Rabbit or fetal calf
α _{2B} AR	Rabbit	1:400	1:50	sc-10723	Santa Cruz	Goat or fetal calf
α _{2C} AR	Goat	1:400	1:50	sc-1480	Santa Cruz	Fetal calf
β ₁ AR	Rabbit	1:400	1:100	sc-568	Santa Cruz	Goat or fetal calf
β ₂ AR	Rabbit	1:400	1:50	sc-9042	Santa Cruz	Goat or fetal calf
β ₃ AR	Goat	1:400	1:50	sc-1473	Santa Cruz	Rabbit or fetal calf
Mouse IgG	Goat	1:3,000	1:100	A-3562	Sigma	–
Rabbit IgG	Goat	1:1,000	1:50	D-0487	Dako	–
Goat IgG	Rabbit	–	1:50	A-4187	Sigma	–
Goat IgG	Rabbit	1:2,500	–	172-1037	Biorad	–

WB Western blotting, IHC immunohistochemistry

vinylidene fluoride membranes (Millipore) were exactly as described (Pradidarcheep et al. 2008). After blocking, the membranes were exposed overnight to the antisera at the concentrations described in Table 1. Antibody binding was visualized with horseradish-peroxidase-conjugated goat antirabbit or goat antimouse secondary antibody and chemiluminescent imaging.

Results

Distinct staining patterns, including low background staining

The staining patterns of five MR and nine AR subtype antibodies in the organs of the rat lower urinary tract, e.g., the ureter (Fig. 1), and the gastrointestinal tract, e.g., the stomach (Fig. 2), were distinct for each antiserum. Thus, the cellular staining patterns were different for all MR antisera. Although the α_{1A} - and α_{1B} -AR staining patterns of the ureter were similar, these antisera produced distinct staining patterns in the stomach. Conversely, the α_{1B} -, α_{1D} -, and α_{2A} -AR antisera produced similar staining patterns in the stomach but distinct patterns in the ureter. These findings demonstrate that each of the antisera generated a staining pattern that differed from all other antisera, with little or no background noise. Unfortunately, such “pretty” staining patterns do not, as we will see, predict specificity for the cognate receptor.

Demonstration of lack of specificity

Western blots with extracts of organs of the lower urinary tract, gastrointestinal tract, and brain were prepared but the outcome was not satisfactory because all antisera yielded too many bands and did not allow specific detection of a major band likely to represent the target receptor (not shown). Whole-cell extracts of cell lines each stably expressing cloned human MR or AR subtypes were used for further evaluation of the various antisera. While, similar to the tissue sections, each MR antiserum generated a distinct “finger print” on the Western blot, that pattern was similar in cell lines expressing the cognate receptor subtype or related subtypes (Fig. 3). Similar observations were made with antisera supposedly acting on α_{2B} -, β_2 -, or β_3 -AR (Fig. 4). These antisera, especially the α_{2B} - and β_3 -AR preparations, produced a distinct banding pattern for Rat-1 fibroblasts (α_{1A} -, α_{1B} -, and α_{1D} -AR lanes), HEK-293 cells (α_{2A} -, α_{2B} -, and α_{2C} -AR lanes), and CHO cells (β_1 -, β_2 -, or β_3 -AR lanes) rather than a unique band (or set of bands in case posttranslational modifications had occurred) that was specific for the receptor that was transfected.

Discussion

In our immunohistochemical experiments, all MR and AR antisera tested showed tissue- and antiserum-dependent staining patterns with a very satisfactory signal-to-background ratio in distinct tissues (Figs. 1 and 2). While

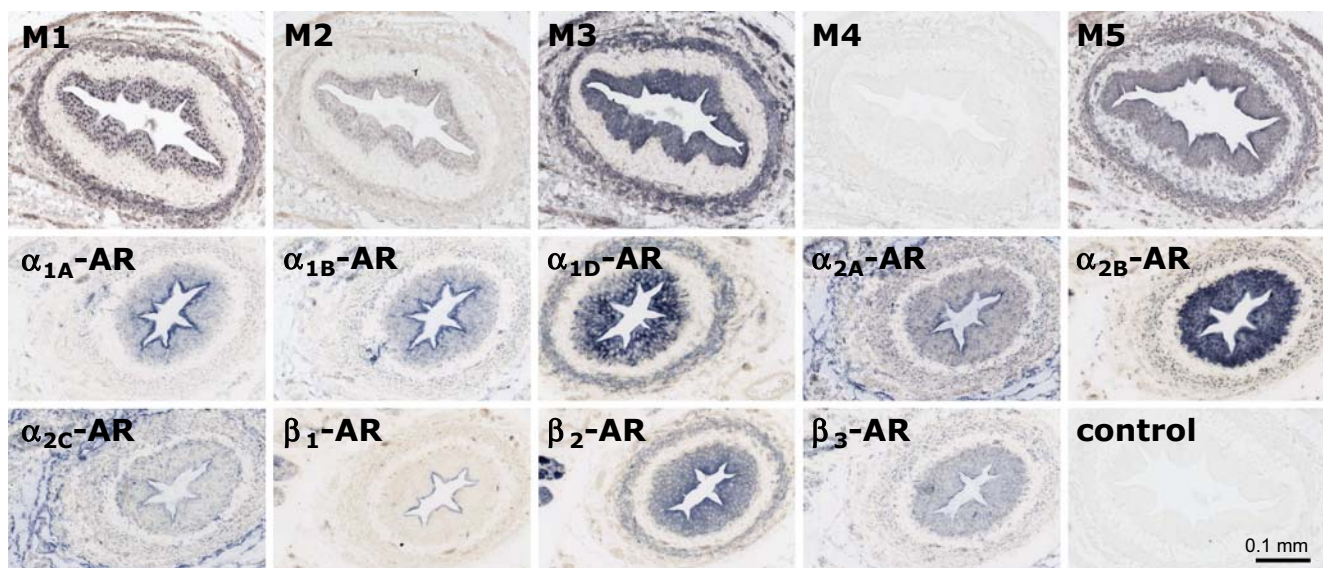


Fig. 1 Staining for the presence of MR and AR subtypes in the ureter of the rat. Control: section incubated without primary antiserum. Bar=0.2 mm

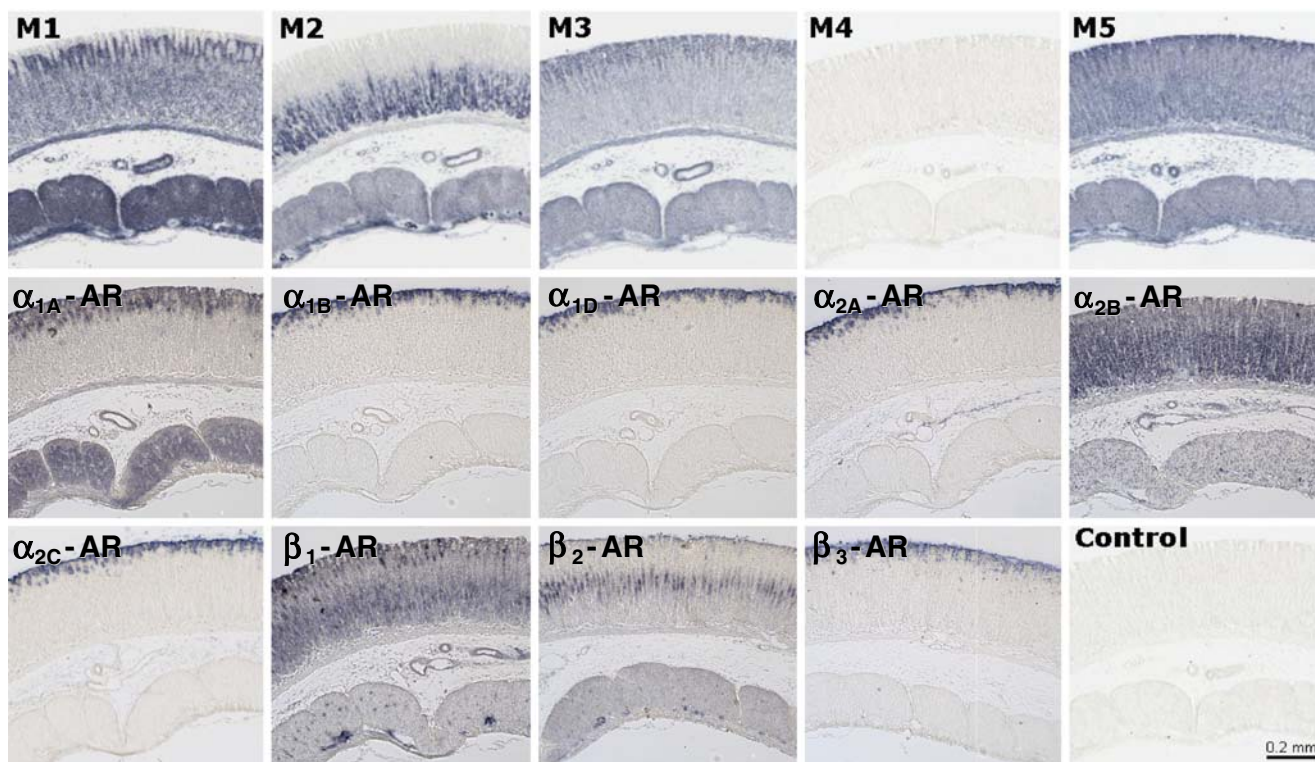


Fig. 2 Staining for the presence of MR and AR subtypes in the glandular stomach of the rat. *Bar*=0.2 mm

each of the antisera exhibited a distinct pattern of bands for each host cell line, i.e., Rat-1 vs. CHO vs. HEK-293, in Western blots, none of them produced a distinct band in the cells expressing their target receptor as compared to those expressing related subtypes. While these findings raise questions about the selectivity of the antisera for their respective cognate receptors, several caveats need to be considered.

Firstly, it is possible that a given receptor presents differently in its native conformation (as seen in immunohistochemistry) as compared to the conformation adopted under denaturing condition (as seen on a SDS gel). While this means that an antibody lacking specificity on Western blots does not necessarily lack specificity in immunohistochemistry, our data nevertheless clearly do not support the idea of specificity. Moreover, transfected cells can of course

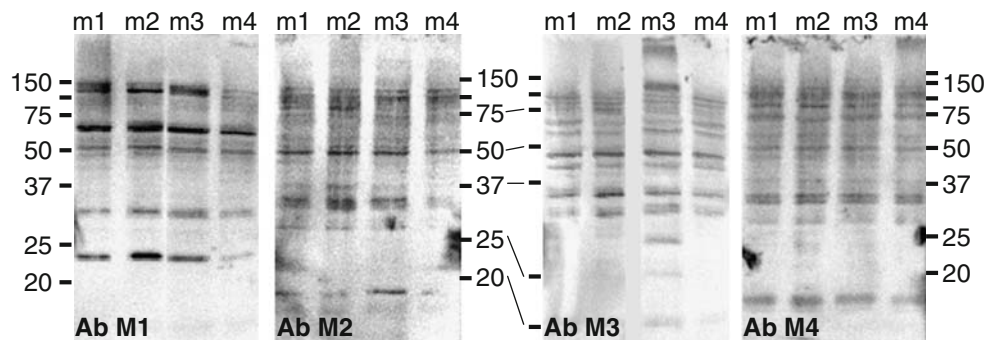


Fig. 3 Staining pattern of antisera against MR subtypes on Western blots of cell lines each expressing one MR. Extracts (50- μ g protein) of cell lines stably transfected with an expression vector encoding the MR indicated above each lane were electrophoresed, blotted, and stained for the presence of MR subtypes with the antisera. The

calculated molecular weights for M_1 , M_2 , M_3 , and M_4 receptors are 51, 52, 66, and 53 kDa, respectively. Note that each antiserum generated a unique staining pattern but that the pattern was identical irrespective of which MR subtype was expressed in the cell line

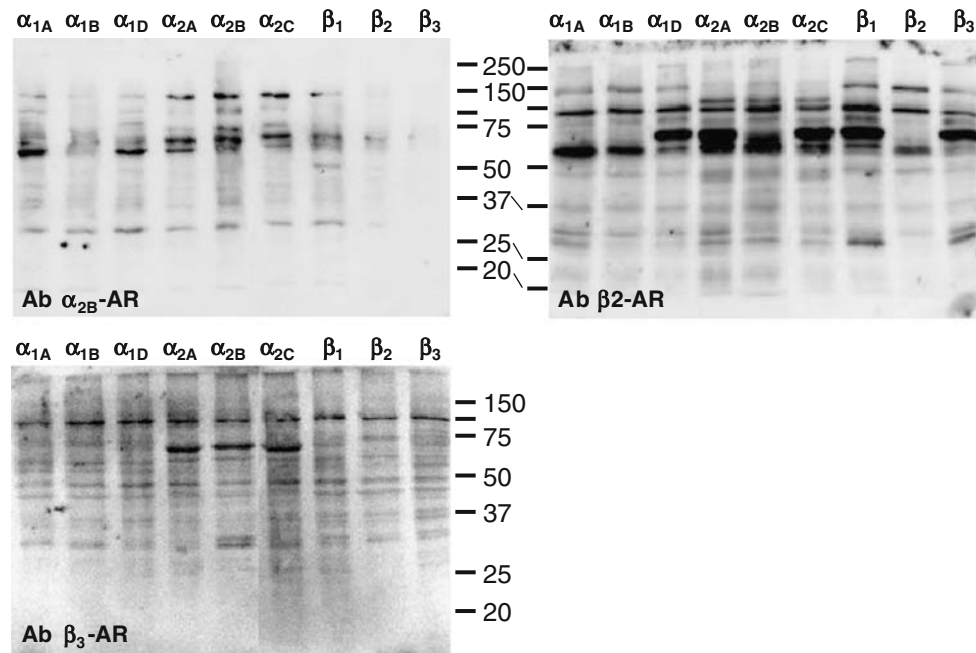


Fig. 4 Staining pattern of antisera against AR subtypes on Western blots of cell lines each expressing one AR. Extracts (50- μ g protein) of cell lines stably transfected with an expression vector encoding the AR indicated above each lane were electrophoresed, blotted, and stained for the presence of the α_{2B} -, β_2 -, and β_3 -AR subtypes with the antisera. The calculated molecular weights for α_{1A} -, α_{1B} -, α_{1D} -, α_{2A} -,

α_{2B} -, α_{2C} -, β_1 -, β_2 -, and β_3 -AR are 52, 56, 59, 50, 50, 50, 50, 47, and 43 kDa, respectively. Note that each antiserum shown generated a unique staining pattern but that none identified a band of the anticipated size and that many bands were shared irrespective of which AR subtype was expressed

also be used to test antibody specificity in immunocytochemistry. Secondly, our comparisons have not been based on non- or mock-transfected cells but rather on those transfected with related receptor subtypes. We actually consider this to be a strength of our approach as a given receptor upon overexpression may appear selective but loses that “selectivity” when studied at the lower expression levels occurring physiologically. This problem is circumvented by the use of the same cell line expressing related subtypes at a similar density. Thirdly, our immunoblots (based upon whole-cell extracts) show numerous bands for each antiserum, whereas those shown in a supplier’s catalog often show a single band only. While the number of bands due to low-affinity binding to other targets depends on the specific experimental conditions, any “specific” antibody should at least identify a unique major band even if the chosen conditions allow detection of various non-specific bands. However, that was not the case for any of the antibodies we have studied. Finally, the possibility should be considered that a given cell line has lost expression of the transfected receptor. While this is always a theoretical possibility, it is extremely unlikely that this applies to all 13 cell lines tested here. Moreover, we have verified the maintained presence of the transfected gene by cell signaling and/or radioligand binding studies for most cell lines within few months of the Western blot experiments.

In summary, we have shown that many receptor antibodies lack specificity for their cognate receptor in Western blots despite yielding very distinct staining patterns in tissues. We conclude that traditional criteria for antiserum specificity such as use of “pre-absorption,” elimination of staining by pre-incubation with the antigen, or the presence of only a single band on Western blots do not reliably predict specificity. While the use of corresponding knockout animals or the presence of identical staining patterns of antisera raised against distinct epitopes are the most reliable tests of specificity, we propose that the use of cell lines transfected with the target as compared to related receptors is a useful alternative to explore the specificity of antisera. We further propose that antisera, which pass one or both hard criteria and have this documented in the specification sheets, be identified as “validated” for easy recognition. It should be kept in mind, of course, that validation only assures the user that such an antiserum recognizes the macromolecule against which it was raised. Nevertheless, even a validated antiserum can, if applied improperly, still produce artifacts. The introduction of validated antisera could, nevertheless, provide the end user with a reliable starting preparation and, thus, avoid many fruitless and costly general confirmation assays that should have been carried out by the supplier. Finally, validated antisera would also further science by diminishing the scientific noise

generated by erroneous data like those exposed in this series of articles.

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