

J.-L. Guéant*
E. Mata*
F. Namour*
A. Romano*
I. Aimone-Gastin
G. Kanny
D.A. Moneret-Vautrin
M.-C. Laxenaire

Criteria of evaluation and of interpretation of Sepharose drug IgE-RIA to anaesthetic drugs

Authors' affiliations:

J.-L. Guéant*, E. Mata*, F. Namour*, I. Aimone-Gastin, Laboratory of Cellular and Molecular Pathology in Nutrition (EP-CNRS 616), University of Nancy, Nancy, France
A. Romano*, Department of Internal Medicine and Geriatrics, UCSC-Allergy Unit, C.I., Colombus, Rome and IRCCS Oasi Maria S.S., Troina, Italy
G. Kanny, D.A. Moneret-Vautrin, Department of Medicine D, University Hospital Center of Nancy, Nancy, France
M.-C. Laxenaire, Department of Anesthesiology, University Hospital Center of Nancy, Nancy, France

Correspondence to:

J.-L. Guéant
Laboratory of Cellular and Molecular Pathology in Nutrition (EP-CNRS 616)
University of Nancy
Nancy
France

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The detection of antidrug specific IgE in serum is usually performed by a sandwich-type immunoassay in which the serum IgE is first adsorbed to a reactive phase and subsequently quantified via the binding of an anti-IgE tracer. The preparation of a new drug-reactive phase requires one to establish carefully different steps of validation:

- 1) criteria of positivity of control sera
- 2) competitive inhibition assays with the soluble drug, which should include the determination of the inhibition constant rather than estimation of a single inhibition percentage, especially when the assay is performed for the identification of determinants
- 3) estimation of nonspecific binding of IgE to the solid phase, including hydrophobic binding.

The competitive inhibition depends on the concentration of the competitor and of IgE in the test-tube and the concentration of reactive drug bound to the solid phase. We have improved the inhibition assay by performing the Dixon test for calculating the inhibition constant (K_i) of the competitor. The K_i of six different muscle relaxants was determined in 12 patients who experienced an anaphylactic reaction to muscle relaxants. The values ranged between 1.5 nM and 2.5 μ M. This confirmed the great heterogeneity of drug IgE cross-reactivity among patients. The K_i value of the incriminated drug was the lowest (affinity, the highest) in eight of the 12 patients. It was better correlated to clinical data than the classical inhibition assay. A hydrophobic environment seemed to be necessary, close to the quaternary ion, to allow IgE binding to the muscle relaxant. By contrast, in tiemonium, a hydroxyl group present at a distance of about 3 Å from the quaternary ion may explain why this molecule had a high K_i (μ M). In conclusion, it should be recommended, in

* These first authors contributed equally to this paper and are listed by alphabetical order.

molecular-recognition studies, that the inhibition constant of the soluble drug and of the related compounds be determined to complement the experiments based only on hapten inhibition assays.

The determination of anti-drug IgE in serum is complementary to skin testing, in diagnosing drug allergy. It has been proved to be an efficient diagnostic tool, particularly in allergic reactions to β -lactams and anaesthetics. The advantages of IgE assays are the absence of risk of either systemic reaction or sensitization and the fact that serum samples can be withdrawn at the time of the adverse reaction and stored for additional testing. This is particularly useful in the case of adverse reactions to anaesthetics. The IgE-RIA has also been applied to study the cross-reactivity of IgE to drugs and related compounds, in investigation laboratories.

The detection of anti-drug specific IgE in serum is usually performed by a sandwich-type immunoassay in which the serum IgE is first adsorbed to a reactive phase and subsequently quantified via the binding of an anti-IgE tracer. The reactive phase is prepared by coupling covalently a drug derivative to a solid phase such as nitrocellulose membrane or polymers (Sepharose, polyacrylamide, polystyrene) (1-4). Coupling reactions are performed with agents such as epoxiranes, cyanogen bromide, or carbodiimide (1-6). The drug derivative is either the soluble drug compound, a compound which is structurally related to the drug (3), or a hapten which is coupled to a carrier protein or a polypeptide (7-9).

The preparation of a new drug-reactive phase requires one to establish carefully different steps of validation:

- 1) criteria of positivity established with negative and positive control sera
- 2) competitive inhibition assays with the soluble drug, which should include the determination of the inhibition constant rather than estimating a single inhibition percentage, especially when the assay is performed for the identification of determinants
- 3) estimation of nonspecific binding of IgE to the solid phase.

These criteria are summarized in Table 1.

About 60% of the life-threatening allergic reactions to anaesthetic drugs are due to muscle relaxants (10). The incidence of reactions to the different muscle relaxants is correlated with their clinical use, except for suxamethonium (10). By coupling alcuronium and D-tubocurarine to epoxy-Sepharose, Baldo & Fisher were the first to perform an IgE

radioimmunoassay which established the IgE-mediated mechanism of the reaction (11). Nonspecific binding of IgE to the solid phase was estimated by coupling either ethanolamine or D-galactosamine, instead of the drug, to the solid phase (12). Competitive inhibition assays with various muscle relaxants and other quaternary ammonium compounds demonstrated cross-reactivity of the IgE of the sensitized patients with all muscle relaxants and the involvement of the quaternary ammonium in the epitope recognition. The cross-reactivity was extended to other drugs containing ammonium such as neostigmine, morphine, pentolinum, and thiopentone trimethaphan (11-13). Unlike β -lactams, the muscle relaxants can generate IgE-mediated histamine release without protein haptenization (14). In fact, these compounds are bivalent molecules which may bridge two IgE, as they contain either two quaternary

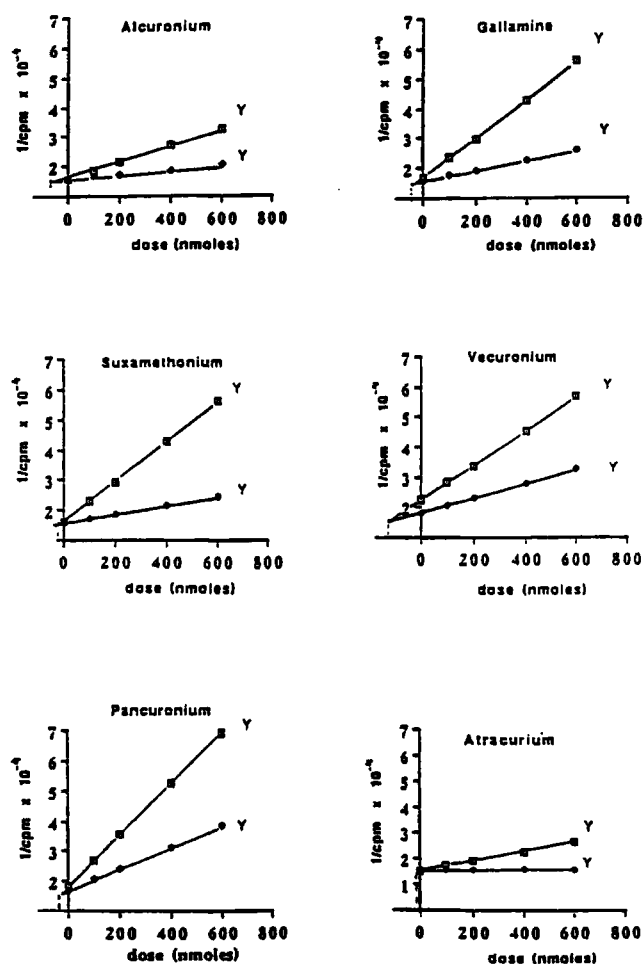


Figure 1. Example of Dixon plots of hapten inhibition IgE-RIA performed with, respectively, 25 (Y₁) and 100 μ l (Y₂) of quaternary ammonium Sepharose (solid phase). Inhibition constants (K_i) of six muscle relaxant drugs were calculated for each tested serum from sensitized patients. They corresponded to x-intercept of two regression curves [Y₁, $\square$; Y₂, $\bullet$].

Table 1. Criteria of evaluation and of interpretation of Sepharose drug IgE-RIA to anaesthetic drugs

Validation/interpretation criteria	Solid phase	Sample	Parameter	Expected result
Limit of positivity	Drug Sepharose	Control sera (n = 10)	Percent uptake	m + 2 SD (positive limit)
	Drug Sepharose	Patient sera	Percent uptake	> m + 2 SD
			Ratio patient/control	2 < 3 (±)
				> 3 (±)
Specific epitope recognition	Drug Sepharose	Patient sera incubated with soluble drugs and related compounds	Inhibition percentage	> 20%, dose-related
			Dixon plot, K_i	$K_i > \mu\text{M}$
Nonspecific uptake	Sepharose-ethanolamin	Patient and control sera	Percent uptake	< positive limit
	Drug Sepharose	High IgE level sera	Percent uptake	< positive limit
Nonspecific uptake	Phenyl Sepharose	Patient sera	Nonspecific hydrophobic-IgE	< positive limit
		Patient sera plus ethylenglycol	Inhibition percentage	< 20%

m=mean value.

ammonium ions or one quaternary ammonium ion and protonated tertiary nitrogen. The cross-reactivity of muscle relaxants has been documented not only with IgE-RIA but also with skin testing and *in vitro* leukocyte histamine release. Despite systematic cross-reactivity in IgE-RIA (3), it is noticeable that some patients may have negative skin tests for some muscle relaxants (15). The same phenomenon may be observed by *in vitro* leukocyte histamine release (16, 17). This heterogeneity of cross-reactivity in the function of the test suggests that the epitope recognition involves both the quaternary ammonium and a neighbouring region, which varies for each patient (3, 12, 18, 19). In contrast to β -lactam allergy, IgE-RIA to muscle relaxants remains positive several years after the accident (20). The incidence of positive IgE-RIA in a reference population seems to be higher than that of the skin reactivity. This discrepancy remains unexplained (21).

The sensitivity of the IgE-RIA is low when coupling muscle relaxants to epoxy Sepharose. It has been improved up to 90% by coupling an analogue of choline to Sepharose via an ether linkage (QAS-RIA) (3). Another commercially available solid phase has also been found to be efficient (22). Seric IgE from patients sensitized to thiopentone may be responsible for *in vitro* cross-reaction with quaternary ammonium IgE-RIA, as one of the IgE determinants involves the ring nitrogen in the pyrimidine nucleus of the molecule (23, 24). IgE-RIA has been also performed with a drug-Sepharose solid phase for patients sensitized to opioids and codeine (25).

Finally, there are several common features in the data of the literature dedicated to the RIA of IgE from patients allergic to β -lactams and to anaesthetics. The sensitivity of these assays has been improved by modifying either the coupling procedure of the solid phase, or the use of a carrier protein or a spacer; it has been estimated with a patient series selected by clinical criteria and skin testing (1–3). It should be interesting to compare the sensitivity of skin tests and IgE-RIA in patients selected with provocation tests. In IgE-RIA, the anti-drug IgE may recognize various determinants of the solid reactive phase, in a variable chemical environment. This heterogeneity of the binding of IgE to the solid phase limits the establishment of clear rules of epitope recognition and cellular reactivity. In addition, determinants have often been identified only by performing inhibition IgE-RIA. In such assays, the competitive inhibition depends on the concentration of IgE in serum and the concentration of reactive drug bound to the solid phase, assuming that no unspecific adsorption of the competitor to the solid phase occurs (5). In addition, the inhibition percentage is not representative of the affinity of the IgE binding to the drug. This may explain why inhibition assays are not correlated to the *in vitro* cell reactivity and to the skin reactivity, at least in patients allergic to muscle relaxants (3). For this reason, we have improved the inhibition assay by performing the Dixon test to determine the inhibition constant (K_i) of the competitor. The K_i is

Table 2. Skin testing and biological data of 12 cases of allergy to muscle relaxant drugs

Patient no.	Muscle relaxant incriminated in adverse reaction	Dilution of positive intradermal reaction					QAS-RIA	Drug tested	Leukocyte histamine release	
		Suxa.	Pancu.	Vecu.	Galla.	Alcu.			% release	dilution
1	Suxamethonium	10 ⁻³	10 ⁻¹	10 ⁻¹	10 ⁻²	—	18.5%	Suxa.	17	10 ⁻²
	Gallamine									
2	Vecuronium	10 ⁻²	10 ⁻²	10 ⁻⁵	—	—	10.4%			
3	Suxamethonium	10 ⁻²	10 ⁻³	10 ⁻¹	—	—	5.7%			
	Pancuronium									
4	Suxamethonium	10 ⁻³	—	—	10 ⁻³	10 ⁻²	14.5%	Suxa.	0	
5	Suxamethonium	10 ⁻³	10 ⁻¹	—	—	—	6.2%	Suxa.	0	
	Vecuronium									
6	Pancuronium	10 ⁻²	10 ⁻³	10 ⁻³	10 ⁻²	10 ⁻³	13.6%	Vecu.	41	10 ⁻²
	Vecuronium							Pancu.	100	10 ⁻²
7	Suxamethonium	10 ⁻¹	10 ⁻¹	10 ⁻³	10 ⁻¹	10 ⁻¹	10.2%	Vecu.	34	10 ⁻²
	Vecuronium									
8	Suxamethonium	10 ⁻³	10 ⁻¹	10 ⁻¹	10 ⁻²	—	5.6%	Suxa.	100	10 ⁻²
	Vecuronium									
9	Alcuronium	10 ⁻³	10 ⁻¹	10 ⁻¹	10 ⁻³	10 ⁻³	33.9%	Alcu.	14	10 ⁻²
	Gallamine									
10	Alcuronium	10 ⁻³	10 ⁻¹	10 ⁻¹	10 ⁻³	10 ⁻²	3.3%			
	Gallamine									
11	Vecuronium	10 ⁻²	—	—	10 ⁻³	—	10.2%	Galla.	35	10 ⁻²
	Gallamine									
12	Gallamine	10 ⁻³	—	—	10 ⁻³	10 ⁻²	3.5%	Galla.	0	

representative of the affinity of drug-IgE binding. The lowest is the K_i , and the highest is the affinity. The reaction can be summarized as follows, using the QAS-RIA as an example:



$$\text{with } K_i = \frac{[\text{drug}][\text{IgE}]}{[\text{drug} - \text{IgE}]}$$

The method of Dixon, which is used for determining the K_i of competitive inhibitors of enzymatic reactions, has been applied to the IgE-RIA, considering the soluble drug as a competitive inhibitor and assuming that the amount of IgE bound to the solid phase is directly related to the detection of the anti-IgE tracer. The K_i of six different muscle relaxants has been determined in 12 patients. The diagnosis of muscle relaxant allergy was established from clinical reports, positive skin testing, positive *in vitro* leukocyte histamine release, and positive QAS-RIA. The clinical and biological data are summarized in Table 2. Two series of inhibition RIA were performed with, respectively, 25 (Y1) and 100 μl (Y2) of solid reactive phase. The serum (50 μl) was preincubated with an increasing concentration of soluble

drug (100, 200, 400, and 600 nanomoles) before the addition of the solid phase and of the tracer, as previously described (3). Finally, the radioactivity and the concentration of the soluble drug (competitor) were plotted, as shown in Fig. 1. The K_i corresponded to the x-intercept of the two regression curves obtained from the Y1 and Y2 IgE-RIA series.

The K_i values calculated for the six muscle relaxant drugs with the 12 patients' sera are given in Table 3. The values ranged between 1.5 nM and 2.5 μM . This confirmed the great heterogeneity of drug IgE cross-reactivity among the patients. Presently, we found that the K_i value of the incriminated drug was the lowest (corresponding to the highest affinity) in eight of the 12 patients. It was better correlated to clinical test data than the classical inhibition assay. In some cases, a 10²–10³-fold difference of affinity was found among the six drugs (patients 2, 4, 8), whereas the difference was limited to less than 10-fold in most of the cases (patients 1, 3, 5, 6, 10, 11, 12). These data confirm that not only quaternary ammonium, but also other regions of the drug are implicated in the IgE binding. A hydrophobic environment seems to be necessary, close to the quaternary ammonium ion, to allow the IgE binding to the neuromuscular binding drugs (NMBD). By contrast, in tiemo-

Table 3. Inhibition constant of soluble muscle relaxants for IgE-quaternary ammonium Sepharose binding, determined by Dixon plot in 12 sensitized patients (see clinical and biological data in Table 2)

Patient no.	Muscle relaxants incriminated in adverse reaction	Inhibition constant (K_i) (nmoles/l)					
		Suxa.	Pancu.	Vecu.	Galla.	Alcu.	Atrac.
1	Suxamethonium	43.1	100.9	40.7	12.9	0	0
	Gallamine						
2	Vecuronium	58.5	4.6	0.5	2.8	348.7	25.5
3	Suxamethonium	54.5	81.4	109.5	160.5	0	0
	Pancuronium						
4	Suxamethonium	330.7	97.7	261.2	28.9	2461.0	8.1
5	Suxamethonium	208.4	168.5	134.5	139.4	159.7	132.9
	Vecuronium						
6	Pancuronium	57.7	38.3	74.5	67.4	390.3	206.6
	Vecuronium						
7	Suxamethonium	8.2	29.6	118.1	23.7	6.3	0
	Vecuronium						
8	Suxamethonium	33.8	5779.0	147.8	1995.3	129.1	82.3
	Vecuronium						
9	Alcuronium	64.6	39.7	74.7	40.6	78.8	4.8
	Gallamine						
10	Alcuronium	155.9	281.3	316.9	345.4	39.8	221.3
	Gallamine						
11	Vecuronium	149.3	0	71.0	70.1	72.6	56.1
	Gallamine						
12	Gallamine	110.9	0	61.1	150.3	181.5	42.4

nium, a hydroxyl group present at a distance of about 3 Å from the quaternary ion may explain why this molecule has a high K_i (μM). Its affinity for specific anti-NMBD IgE is therefore much lower than that usually observed for NMBDs. In addition, we have noticed that IgE-RIA with tetraethylammoniummethyl-trihydroxymethyl-Sepharose (TEAE)-THM was negative in 21 sera with a positive QAS-RIA (18). This TEAE-THM has a steric structure which shares quaternary ammonium and tertiary ammonium ions in a direct hydrophilic environment.

The use of the Dixon plot should be extended to the inhibition assays which are performed for identifying new IgE-binding determinants of drug compounds such as anaesthetics and β -lactams, as it allows one to estimate the affinity of the IgE-drug binding reaction and avoids artefactual nonspecific inhibition due to nonspecific binding of the competitor to IgE. The determination of the K_i could also help in eliciting compounds to be used in perfusion of monohaptens for inhibiting the cell mediator release in sensitized patients.

One of the nonspecific interactions which may occur between seric IgE and the solid phase is hydrophobic binding

(26). This may give false positive results, as it produces nonspecific uptake of IgE to the solid phase and even artefactual results of inhibition assays. We have used either phenyl-Sepharose or octyl-Sepharose for detecting hydrophobic IgE in serum by RIA. Phenyl-Sepharose IgE-RIA was at $4.6 \pm 0.7\%$, $12.5 \pm 6.5\%$, and $17.87 \pm 8.9\%$ in control subjects ($n=24$), in atopic patients with positive Phadiatop[®] ($n=30$), and in patients with drug allergy ($n=23$), respectively (26).

Hydrophobic IgE has also been described in the serum of patients who reacted to Diprivan, a hypnotic drug which contains propofol in a phospholipid emulsion (27). Harle & Baldo proposed the benzyl side-chain as a possible epitope involved in allergic reactions to penicillin (28). In fact, we observed a significant inhibition of IgE-RIA to penicillin with nonrelated compounds which contained a cyclohexenyl ring. A high level of hydrophobic IgE (with phenyl-Sepharose IgE-RIA higher than 20%) in the serum of a patient allergic to a hydrophobic drug may be a risk factor for IgE cross-reactivity to another drug (18, 26). We have reported one case of adverse reaction to anaesthetics in favour of such a hypothesis. Initially, the patient was

allergic to penicillin with positive RAST-IgE and positive skin tests. He presented later a giant urticarial lesion after an intravenous injection of Diprivan. The serum produced a highly positive phenyl-Sepharose IgE-RIA, and cross-reactivity between penicillin and Diprivan was found by inhibition assay. Such a reaction could be the result of nonspecific hydrophobic drug-binding with IgE, rather than a classical allergic reaction via a monospecific anti-drug IgE.

In conclusion, drug solid-phase IgE-RIA is a useful diagnostic tool for allergy to anaesthetics. However, the method has to be validated in order to estimate the threshold value of positivity. It should be recommended, in molecular-recognition studies, that the inhibition constant of the soluble drug and of the related compound be determined to complement the experiments based on hapten inhibition assays. The use of IgE-RIA for identifying the molecular determinants of IgE binding needs also to take into account the hydrophobic binding of IgE to the solid phase.

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