

Original Article

IgE-Mediated Allergy to Pholcodine and Cross-Reactivity to Neuromuscular Blocking Agents: Lessons from Flow Cytometry

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Background: Immunoglobulin E antibody-mediated allergic reactions to opioids are rare and difficult to document correctly.

Objective: Assessment of the basophil activation test in the diagnosis of IgE-mediated allergy to the antitussive pholcodine and associated sensitizations to neuromuscular blocking agents (NMBA).

Methods: Three patients with a suspected IgE-mediated allergy to pholcodine were investigated using skin tests, quantification of specific IgE, and flow cytometric activation of basophils.

Results and conclusion: Flow cytometric activation of basophils, with simultaneous analysis of CD63 appearance and median histamine content per cell, is the only technique capable to correctly document pholcodine allergy. The negative predictive value of basophil activation tests might help to elucidate on the controversial putative cross-reactivity between pholcodine and NMBA. © 2013 International Clinical Cytometry Society

Key terms: IgE antibodies; pholcodine; opioids; neuromuscular blocking agents; anaphylaxis; basophil activation; histamine

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Despite the wide use of opioids, immunoglobulin E antibody (IgE)-mediated allergic reactions to opioid analgesics and related antitussives remain highly anecdotal (1). Pholcodine (3-(2-morpholinyl-ethyl) morphine) is an opioid antitussive indicated in the symptomatic treatment of unproductive coughs in children and adults. For decades, pholcodine-containing medicines have been available as syrups, lozenges, suppositories, and oral capsules and can currently be purchased either by prescription or as over-the-counter medicines in different EU member states (European Medicines Agency: www.ema.europa.eu). However, to our knowledge no well-documented cases of immediate IgE-mediated allergy that occurred within 1 h after intake of the antitussive have been described. In 2005, Codreanu et al. (2) reported on a patient suffering from a facial angioedema 8 h after intake of a pholcodine-containing syrup in which diagnosis of pholcodine allergy was based upon skin tests and an open oral challenge with a laryngeal

angioedema 9 h after a cumulative dose of 20 mg. However, basophil activation test and leukotriene release test were negative. Florvaag et al. (3,4) described three individuals with late-onset (4–7 days) reactions that were related to the intake of pholcodine. In these patients, respectively suffering from generalized pruritus, a mild localized urticaria and eyelid edema, diagnosis was based only upon a rise of specific IgE (sIgE) to pholcodine. Alternatively, it has been suggested that pholcodine may sensitize and booster already sensitized individuals to synthesize IgE antibodies to epitopes like tertiary and quaternary ammonium ions implicated in anaphylaxis

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from neuromuscular blocking agents (NMBA) (3–6). However, the mechanism(s) for such a selective sensitization through pholcodine, that structurally differs only from morphine and pholcodine by its morpholinyl-ethyl-3 group at position 3, remain(s) obscure (1,7,8). Alternatively, although the clinical significance of these sIgE antibodies to NMBA remains entirely undetermined and controversial, it led to the withdrawal of the antitussive in Norway (March 2007) (9).

As reviewed recently elsewhere (1), correct diagnosis of IgE-mediated opioid allergy is fraught by difficulties that mostly relate to the unavailability of validated opioid drug-specific IgE assays and uncertainties associated with skin testing with potent histamine-liberators by cutaneous mast cells (10). Moreover, for many years, codeine has been a positive control for skin prick testing (SPT) (11) and SPT has been argued to be of no value in opiate sensitivity (12). In contrast, opioids such as morphine and diamorphine (heroin) and buprenorphine seem not to trigger histamine release from human basophils (13,14). Alternatively, pholcodine-specific IgE antibodies have been demonstrated in not less than 6% of blood donors from Norway (15) where the antitussive was over-the-counter available until 2007. Specific IgE to pholcodine was also frequently detected in patients with a high total IgE and in almost all patients suffering from rocuronium allergy (16).

Here we describe three patients with generalized hypersensitivity reactions immediately after intake of pholcodine-containing syrups in which an IgE-mediated pathomechanism is suspected from quantification of specific IgE and positive basophil activation tests with analysis of surface markers and intracellular histamine content of individual cells, as described by Ebo et al. (17). Briefly, in this technique intracellular histamine and its release is analyzed flow cytometrically by an enzyme affinity method using the histaminase diamine oxidase conjugated to fluorochromes. Application of this enzyme-affinity technique to quantify intracellular histamine was first described by Dvorak et al., who used diamine oxidase (DAO) coupled to gold particles to study the intracellular localization and content of histamine by electron microscopy (18,19). The Histaflow® technique (17) enables to quantify histamine and its release in a multi-parameter analysis with the quantification of different surface activation markers which might bring new insights to the understanding of the mechanisms that govern the different degranulation profiles.

In view of the concern of pholcodine in eliciting sensitization to NMBA, the patients had additional quantification of sIgE and more functional tests such as skin and basophil activation tests to elucidate on the clinical relevance of eventual IgE findings.

MATERIALS AND METHODS

Study Population

Three patients who had experienced generalized anaphylaxis within 15–60 min upon intake of a pholcodine-

containing syrup and three healthy control individuals without pholcodine allergy were studied. Participants gave a written informed consent as approved by the Ethical Committee of the University Hospital Antwerp (Belgium).

Skin Tests

Skin tests were performed with pholcodine containing cough syrup (Bronchopeptoralis pholcodine®, Medgenix, Brussels, Belgium), morphine (Stellorphone®, Sterop, Brussels, Belgium), codeine (Codeine phosphate 20 mg/mL), rocuronium (Esmeron®; MSD, Brussels, Belgium), vecuronium (Norcuron®; MSD, Brussels, Belgium), atracurium (Tracrium®; GSK, Genval, Belgium), cisatracurium (Nimbex®; GSK, Genval, Belgium), suxamethonium (Myoplegine®; Christiaens, Brussels, Belgium), a negative (saline buffer) and a positive control (10 mg/mL histamine; HAL Allergy Benelux BV, Haarlem, The Netherlands). Concentrations of SPT for pholcodine, morphine, and codeine ranged from 1/1,000 to 1/10 of the stock solution (12). Concentrations of skin tests for NMBA were established according to Mertes et al. (20). SPT and intradermal test (IDT) responses were considered positive when the wheal equaled or exceeded diameters of 3 and 8 mm, respectively. For the SPT and IDT, commercially available drugs were diluted in a physiologic solution immediately before use. For the IDT, injection of 0.05 mL was performed through a hypodermic needle, and reactions were read after 20–30 min by measuring diameters of wheals and flares.

Oral Codeine Provocation Test

An oral codeine provocation test was performed in the patients with a cumulative therapeutic dose of 30 mg codeine to assess the predictive value of the basophil activation experiments and to study tolerability for this popular antitussive and analgesic drug.

Total and Specific IgE

Total IgE, sIgE to rocuronium, atracurium, suxamethonium, morphine, pholcodine, and poppy seed (21) (*Papaver somniferum*) was quantified by an ImmunoCAP system (ThermoFisher Scientific, Uppsala, Sweden). ImmunoCAP rocuronium and atracurium were obtained from ThermoFischer Scientific as an experimental ImmunoCAP prototype made for research use. All assays were performed according to the manufacturers' recommendation.

In Vitro Activation of Basophils

Analysis of in vitro basophil activation was performed as described by Ebo et al. (17). Briefly, 200 µL endotoxin-free heparinized whole blood were challenged at 37°C for 20 min with 200 µL buffer as negative control, 200 µL anti-IgE (Pharmingen, BD Bioscience, Erembodegem, Belgium) as a positive control, and 200 µL of serial dilutions of pholcodine (Fagron, Waregem, Belgium) (1–1,000 µg/mL), morphine (Stellorphone®, Sterop, Brussels, Belgium) (10–100 µg/mL), codeine (Escapo C.V.,

Mechelen, Belgium) (1–1,000 µg/mL), and five NMBAs (rocuronium, vecuronium, atracurium, cisatracurium, and suxamethonium) as described earlier (22). Reactions were stopped by chilling on ice, adding 1 mL ice-cooled PBS-EDTA 10 mmol/L EDTA and spinning for 5 min (4°C, 200g). To select and quantify basophil activation, cells were stained with 20 µL of monoclonal anti-human IgE (clone GE-1, Sigma Aldrich GmbH, Steinheim, Germany) labeled with Alexa Fluor 405 (Molecular Probes, Invitrogen, Paisley, UK) and 10 µL of monoclonal anti-human CD63-FITC (clone H5C6, BD Bioscience), 10 µL CD203c-APC (clone NP4D6, Biologend, San Diego, CA, USA) for 20 min on ice. Cells were lysed/fixed with 2 mL Phosflow Lyse/Fix buffer for 20 min (37°C). Cells were washed with and resuspended in PBS with 0.1% Triton-X-100 (PBS-TX, pH = 7.4). To stain intracellular histamine 10 µL PE-labeled DAO (BD Biosciences, Erembodegem, Belgium) was added and incubated at 37°C (45 min). Cells were washed and re-suspended in PBS with 0.1% sodium azide and measured.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a FACS-Canto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) as described by Ebo et al. (17). Correct compensation settings for the fluorochromes used were performed using BD CompBeads (BD Biosciences). Fluorescence minus one (FMO) and DAO staining without permeabilization was used to set a marker between DAO positive and negative cells. Flow cytometric characterization of basophils relied upon a combination of side scatter (SCC), anti-IgE and CD203c. Standardization of intracellular histamine content was performed using standardized fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech, Lake Forest, IL) as described by the manufacturer. Results were expressed as %CD63 positive basophils and as median histamine release per cell (MHC). MHC expressed as the median fluorescence intensity (MFI/cell) was calculated as the difference between MFI per cell in non-degranulating (CD63⁺ and CD203c^{dim}) basophils minus the MFI per cell in degranulating (CD63⁺ and CD203c^{hi}) basophils.

RESULTS

The demographics, characteristics, and test results for opioids of the patients and control individuals are summarized in Table 1.

Skin Tests

Skin tests with pholcodine, morphine, and codeine were not able to discriminate between patients and control individuals. Actually, for the higher concentrations (1/10 and 1/100) skin tests were positive in patients and controls, whereas for the 1/1,000 dilution tests were negative in patients and controls. Skin tests with five different NMBA (rocuronium, vecuronium, atracurium, cisatracurium, and suxamethonium) were negative in all patients.

Table 1
Patient Demographics and Results of Total and Specific IgE Quantification

	Age (years)	Sex	Allergy	Reaction (history)	Interval (min)	Total IgE (kUa/L)	sigE (kUa/L) pholcodine	sigE (kUa/L) morphine	sigE (kUa/L) poppy seed	sigE (kUa/L) rocuronium ^a	sigE (kUa/L) suxamethonium ^a	sigE (kUa/L) atracurium
PT1	55	M	OAS	U, AE, DC	15	6500	25.9	100	<0.10	8.36	1.96	<0.10
PT2	39	F	House dust mite	U, AE, DC, B	15	1765	100	100	<0.10	21.2	1.96	<0.10
PT3	60	F	/	U, AE, B	60	75	2.35	0.87	<0.10	0.17	<0.10	<0.10

PT: patient; M: male; F: female; AE: angioedema; B: bronchospasm; DC: decreased consciousness; OAS: oral allergy syndrome; U: urticaria

All specific IgE (sigE) quantifications were negative in the control individuals.

^aNote that for rocuronium and suxamethonium a drug-specific decision threshold of 0.13 kUa/L and 0.11 kUa/L was applied according to Ebo et al. (17).

Specific IgE

As displayed in Table 1, sIgE to pholcodine, morphine, rocuronium, and suxamethonium was positive in all the patients and negative in all the control individuals. Specific IgE to poppy seed (*P. somniferum*) and atracurium was negative in all patients.

Flow Cytometric Quantification of Basophil Activation

Basophil activation tests in patients showed a consistent dose-dependent activation of the cells with pholcodine ranging from 0 to 41% of CD63 positive basophils (Figs. 1 and 2). Median histamine release per cell (MHC/cell) ranged from 0 to 239 MFI/cell. For stimulation with morphine and codeine no basophil activation was demonstrable. Actually, CD63 up-regulation remained negative and no histamine release per cell was measured. In healthy control individuals, the basophil activation tests with morphine, pholcodine, and codeine were negative. The basophil activation tests with different NMBA were negative in all patients as well.

Oral Codeine Provocation Test

An oral codeine provocation test was negative in the patients and entirely mirrored the BAT/HistaFlow® results.

DISCUSSION

IgE-mediated allergic reactions to opioids are anecdotal and difficult to diagnose mostly due to uncertainties associated with skin testing and unavailability of validated opioid drug-specific IgE assays (1,12).

To our knowledge, this is the very first report on three patients with anaphylaxis to pholcodine in which an IgE-mediated mechanism is suggested by quantification of sIgE and by selective positive basophil activation and DAO labeling results. Although one could argue that quantification of sIgE to pholcodine in our patients appears diagnostic, it should be kept in mind that Florvaag et al. found positive sIgE results to pholcodine in up to 10 % of an atopic population (15) and we found clinically irrelevant sIgE antibodies to pholcodine in virtually all patients with a rocuronium allergy (16). Moreover, we have shown that high total IgE titers might interfere with the quantification of pholcodine sIgE (16). Furthermore, higher total IgE titers could result from a boosting effect of this antitussive (3).

Therefore, we performed basophil activation and skin tests in order to study the biological activity and clinical relevance of the pholcodine and morphine IgE antibodies found in our patients. Unlike skin tests, basophil activation tests enabled to correctly diagnose pholcodine allergy in the patients. Using the recently validated HistaFlow technique (17), only pholcodine (but not the structurally closely related opioids morphine and codeine) triggered significant basophil degranulation in patients but not in control individuals. In contrast, skin tests with opioids were confirmed to be absolutely

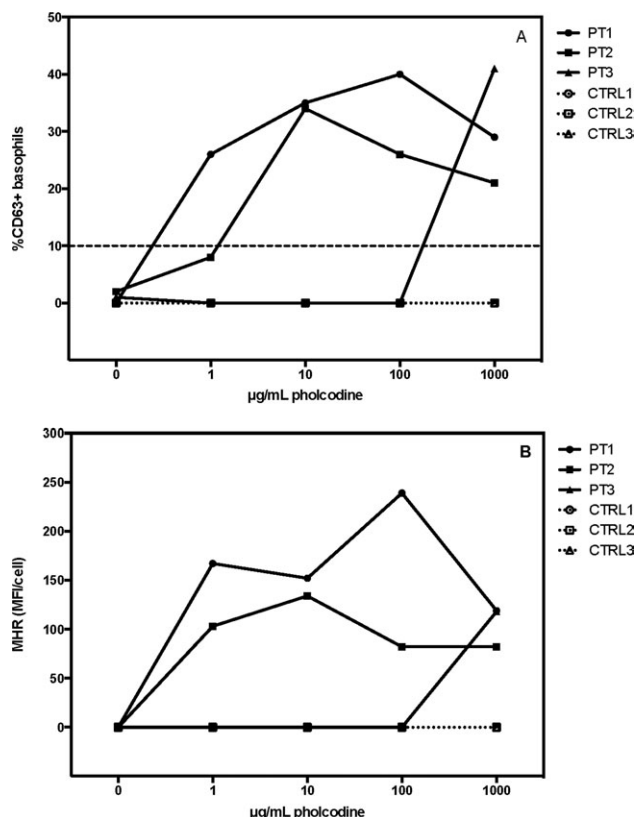


Fig. 1. **A:** CD63 up-regulation on basophils after stimulation with pholcodine. Activation of basophils is expressed as net percentage CD63 positive basophils. Upon challenge with pholcodine, basophils of the patients but not of the control individuals show an up-regulation of CD63 expression from 0 to 41%. The dashed line in the top panel denotes the decision threshold of CD63% up-regulation that is currently arbitrarily set at 10%. **B:** Mean histamine release of basophils after stimulation with pholcodine. Histamine release is expressed as MFI (median fluorescence intensity per cell). Stimulation of basophils with the antitussive results in a clear histamine release per cell that plateaued for the applied concentrations. There is no histamine release in the control individuals.

unreliable to diagnose IgE-mediated opioid allergy (1,12), as they did not discriminate between patients and control individuals tolerant for opioids. Moreover, skin tests failed to predict the negative challenge test with codeine, another popular antitussive that is structurally related to pholcodine and morphine. Finally, unlike Armentia et al. recently published (21), sIgE to *P. somniferum* was found unreliable to diagnose allergy to the opiate pholcodine.

During the last decade, there has been substantial debate about the potential of pholcodine to cause sensitization toward NMBA that are structurally related to the antitussive (23,24). It has been suggested that pholcodine may sensitize and boost already sensitized individuals to synthesize sIgE antibodies to epitopes like tertiary and quaternary ammonium ions that are implicated in anaphylaxis from NMBA (3,4). Moreover, although the absence of firm evidence for clinical relevance of these sIgE antibodies this observation has already led to withdrawal and restrictive prescription of cough suppressant

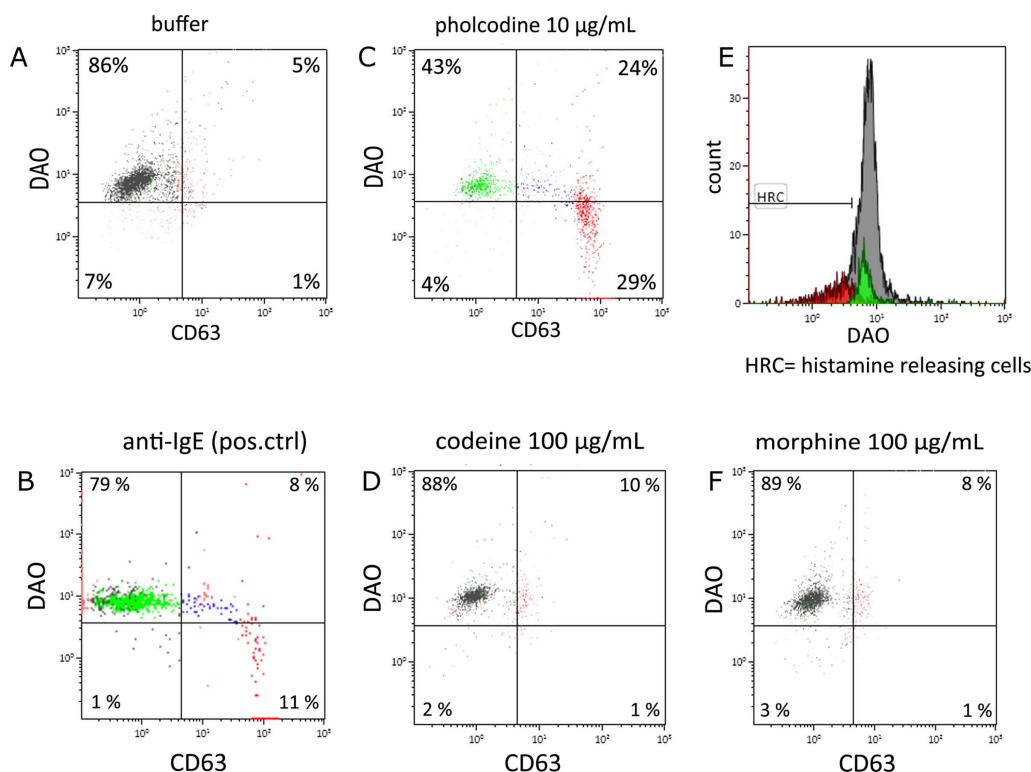


FIG. 2. Representative sample of histamine release after activation of the basophils with buffer (A), anti-IgE as positive control (B), pholcodine 10 µg/mL (C), codeine 100 µg/mL (D), and morphine 100 µg/mL (F). Basophils are characterized using side scatter (SSC), anti-IgE+ and CD203c+ positive cells. Histamine-containing basophils are defined as DAO + cells. Panel (E) shows the DAO overlay histogram (Y-axis panel (A) and (C)). In this sample, after activation with pholcodine, 29% of the basophils demonstrate complete release of their histamine content (DAO) and are situated in the lower-right quadrant of panel (C). [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com.]

in some European countries (9). Our data demonstrate that patients with established pholcodine allergy might indeed show IgE-seroreactivity to NMBA such as rocuronium and suxamethonium. However, the clinical relevance of these serologic findings remains obscure as more functional investigations such as basophil activation tests and skin tests were entirely negative for NMBA. Presumably, this observation is a reflection of the underlying processes in the different tests. The sIgE tests simply measure the interaction between NMBA and the paratope (epitope binding site) of the antibody, whereas basophil activation tests and skin tests depict drug-induced crosslinking of cell-bound sIgE via the paratope and subsequent degranulation with release of the mediators. Therefore, and taking into account our prior observations about clinically irrelevant sIgE to rocuronium (16,25), our current practice is not to absolutely discourage the use of rocuronium and morphine in patients demonstrating positive sIgE but negative skin tests and BAT/HistaFlow results to these drugs. In this context, the negative predictive value of skin tests and the BAT/HistaFlow technique will be studied by challenge tests and/or long-term follow-up studies.

In conclusion, for the first time three patients with an immediate pholcodine allergy are presented in whom only basophil activation proved to enable correct

diagnosis to this opioid. Moreover, it is anticipated that the negative predictive value of the BAT/HistaFlow may be of help to further elucidate on the controversial putative cross-reactivity between pholcodine and NMBA and other opioids.

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