

Anaphylaxis to chlorhexidine. Case report. Implication of immunoglobulin E antibodies and identification of an allergenic determinant

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Summary

Background There are many reports of allergic reactions, including anaphylaxis, following exposure to chlorhexidine. Reactions may occur via contact with the skin and mucous membranes or from catheters treated with the antibacterial agent. Apart from implicating chlorhexidine in immunoglobulin (Ig) E antibody-binding studies on serum from an anaphylactic patient, little work has been done on the molecular basis of recognition of the agent in sensitive subjects. **Objectives** The molecular basis of IgE-binding to chlorhexidine was closely examined with the view of defining its fine structural recognition features by antibodies from a subject who experienced anaphylaxis following contact with the antiseptic.

Methods Tryptase determinations, different drug-solid phases, immunoassays and quantitative hapten inhibition studies with chlorhexidine and selected structural analogues were employed together with serum from the anaphylactic patient. Results were analysed to define the complete drug allergenic determinant and to identify the important structural features complementary to the IgE antibody combining sites.

Results The subject's serum tryptase levels sampled after the reaction were elevated and employment of a chlorhexidine-EA Sepharose solid phase showed the presence of serum IgE antibodies to the drug. Lack of inhibition by 4-chlorophenol and other selected substituted phenyl compounds showed that the terminal groups at each end of the chlorhexidine molecule, alone, did not account for antibody recognition of the antibacterial agent. Although chlorhexidine and alexidine, the structures of which each comprise part of the chlorhexidine molecule, showed significant inhibition of the binding of IgE antibodies to chlorhexidine, neither compound was as potent an inhibitor as chlorhexidine itself. Two molecules of chlorhexidine make up the symmetrical molecule of chlorhexidine while the interior structure of alexidine (that is excluding the terminal 2-ethylhexyl groups) is identical to part of the chlorhexidine molecule.

Conclusions Taken together, for this patient, these results lead to the conclusion that the whole chlorhexidine molecule is complementary to the IgE antibody combining sites and that the 4-chlorophenol, biguanide and hexamethylene structures together comprise the allergenic determinant. Hence, like one of the trimethoprim determinants identified, but unlike most drug allergenic determinants identified so far, the chlorhexidine allergenic determinant identified here encompasses the entire molecule.

Keywords: chlorhexidine allergy, drug allergy, anaphylaxis, allergenic determinant

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Introduction

Reports of adverse reactions of both the immediate and delayed-type following exposure to chlorhexidine are numerous. Anaphylaxis to the compound has been reported after contact with the skin, eyes, and other mucous membranes [1–8] and with catheters treated with the antiseptic [9–11].

Immunoglobulin (Ig) E antibodies that react with chlorhexidine were found in the serum of a subject who collapsed 70 min after treatment of an abraded wound with 0.5% chlorhexidine gluconate in alcohol. Limited specificity studies showed antibody recognition of chlorguanide [2], two molecules of which make up the symmetrical molecule of chlorhexidine.

In this study, detailed quantitative hapten inhibition studies were carried out with chlorhexidine-reactive IgE antibodies identified in the serum of a subject who experienced an anaphylactic reaction while having a cystoscopy under local anaesthesia. Investigations with the drug and selected structural analogues clearly defined the fine structural features recognized by the complementary IgE antibodies.

Case report

A 75-year-old man was referred following three anaphylactic events. The first occurred in September 1995 during general anaesthesia for coronary artery bypass grafts. Ten minutes after induction he developed a marked drop in blood pressure, bronchospasm, tachycardia, and increased pulmonary artery pressure. He settled after metaraminol and i.v. fluids, and his peri-operative course was uneventful.

In July 1996 a transurethral resection of prostate was performed under spinal anaesthetic. At cystoscopy he developed headache, rash and bronchospasm and settled after treatment.

He had a further cystoscopy in February 1998. During that procedure he became flushed, wheezy and hypotensive, and had a cardiac arrest. He was successfully resuscitated. Elevated serum tryptase levels of 60.4 and 26.6 µg/L were found 3.5 h and 9.5 h after the event indicating a true anaphylactic reaction.

There were discussions with medical personnel involved with each episode together with a review of all notes. The only common pharmacological agent was urethral jelly containing lignocaine 2% and chlorhexidine 0.05%. In fact, this was the only drug used during the third procedure.

Two months after the third anaphylactic episode, the following RASTs were performed and all were negative: latex, ethylene oxide, formalin, lignocaine and procaine. Subsequently he had skin prick tests, intradermal tests, and sequential subcutaneous challenges to lignocaine without any positive or adverse effects.

From a clinical point of view, chlorhexidine was the cause of the anaphylaxis because (i) the urethral jelly containing lignocaine and chlorhexidine was the only common drug to the three procedures; (ii) the urethral jelly was the only drug used in the third procedure and; (iii) lignocaine was excluded as a cause because of negative *in vitro*, *in vivo* and challenge tests to this drug.

Skin tests to chlorhexidine were deemed unethical because the patient had developed profound anaphylaxis with cardiac arrest following topical administration of this drug. An *in vitro* method for detecting sensitivity to chlorhexidine was therefore pursued.

Materials and Methods

Drugs and chemicals

Chlorhexidine (Hibitane, ICI, Cheshire, UK) and chlorguanide hydrochloride (Paludrine, ICI) were obtained from the Pharmacy, Royal North Shore Hospital. Alexidine dihydrochloride, aminoguanidine hemisulphate, arginine hydrochloride, human serum albumin (HSA), N,N'-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC), N-hydroxy-succinimide and N,N-dimethylformamide (DMF) were from Sigma Chemical Co. (St Louis, MO, USA). 4-Chloroacetanilide, 4-chloroaniline, 4-chlorophenylhydrazine hydrochloride, 4-chlorophenyl and 4-guanidinobenzoic acid hydrochloride were from Fluka Chemie AG (Buchs, Switzerland). Epoxy activated Sepharose 6B and ECH Sepharose 4B were from Pharmacia (Uppsala, Sweden). ¹²⁵I-antihuman IgE was from Bioclone (NSW, Australia).

Preparation of chlorhexidine–solid phase complexes

In an effort to employ the most reactive drug–solid phase support, four different preparations were developed.

Chlorhexidine-EA Sepharose 6B To a solution of chlorhexidine (120 mg) in distilled water (10 mL), the washed and drained epoxy activated Sepharose 6B (1 g) was added and the pH of the mixture adjusted to 10.0 with 1 M NaOH. The mixture was gently shaken at 25 °C for 20 h and after washing with water, 0.1 M borate buffer pH 8 and 0.1 M acetate buffer pH 4, the remaining free activated groups on the gel were blocked with 1 M ethanolamine pH 9 in distilled water overnight at room temperature. The gel was then washed again as described above, finishing with a final wash of distilled water and resuspended in distilled water and stored at 4 °C.

Chlorhexidine-EA paper discs Paper (Whatman no. 1) discs (0.5 g) were first activated by incubating with 1,4-butanediol diglycidyl ether 70% (bis-oxirane) (7 mL) in 1 M

NaOH containing 3.33 mg/mL of NaBH₄ (3 mL) for 6 h at room temperature with rotation. The reaction was stopped by washing the discs with distilled water. The epoxy-activated discs ($n=40$) were then soaked in a solution of chlorhexidine (20 mg) in distilled water (2 mL), pH 10.0 for 20 h at room temperature with gentle vibration. The discs were washed with distilled water, borate buffer pH 8 and acetate buffer pH 4, then blocked overnight at room temperature with 1 M ethanolamine pH 9. After further washing as above and a final wash with distilled water, the discs were blotted dry and stored at -20°C .

Chlorhexidine-ECH Sepharose 4B The washed and drained gel (from 3 mL of sedimented ECH Sepharose 4B) was added to a solution of chlorhexidine (80 mg) in distilled water (6 mL) previously adjusted to pH 5.5 with 0.1 M HCl. Following the addition of EDC (200 mg) and re-adjustment to pH 5.5, the reaction mixture was incubated at 25°C for 20 h. To remove any remaining uncoupled ligands, the adsorbent was washed alternatively with three cycles of 0.1 M acetate buffer pH 4 followed by 0.1 M Tris buffer pH 8, both containing 0.5 M NaCl. After a final wash with distilled water, the product was resuspended in distilled water and stored at 4°C .

Chlorhexidine-DCC-HSA Chlorhexidine (20 mg) was dissolved in distilled water (2 mL). After the addition of DCC (12 mg) and N-hydroxysuccinimide (14 mg) in DMF (0.5 mL), the mixture was rotated at room temperature for 2 h and the precipitate removed by filtration. HSA (20 mg) was dissolved in the supernatant and the reaction mixture was gently rotated at room temperature overnight. The mixture was dialysed against 50 mM phosphate buffer saline pH 8 for three days with frequent changes. The chlorhexidine-HSA conjugate was then adsorbed to nitrocellulose (NC) discs (20 μg complex per disc for 20 h at room temperature) and stored at -20°C .

Serological methods

Sera from adults allergic to house dust mites and/or pollens were obtained after clinical diagnosis, made on the basis of case history and skin tests, was confirmed by the detection of allergen-specific IgE antibodies in radioallergosorbent tests [12]. Other control sera were obtained from nonallergic subjects with high total serum IgE levels and normal, nonallergic laboratory workers. Cord serum was provided by the Department of Obstetrics, Royal North Shore Hospital, Sydney, and used as an IgE-‘free’ control.

Antibodies were detected as previously described [13,14] with some modifications. Briefly, serum (25 μL) was incubated for 3 h at room temperature with the drug-solid phase. Discs or sedimented Sepharose conjugates were then washed three times with PBS containing 0.05% Tween 20 and 0.05% sodium azide before incubation overnight with about 30 000 c.p.m. of ^{125}I anti-human IgE. After further

washes as above, tubes were counted in a Packard Auto-Gamma Spectrometer. The presence of specific IgE antibodies was determined by the per cent radioactive uptake of ^{125}I anti-human IgE, i.e. per cent of counts added.

In order to facilitate the quantitative comparison between inhibitors with different solubilities and pHs, two sets of solutions prepared in different diluents were studied. Chlorhexidine, aminoguanidine and arginine were dissolved in 10 mM phosphate-buffered saline (PBS), pH 7.5. Guanidinobenzoic acid was also dissolved in the same buffer but needed adjustment with 1 M NaOH to bring the pH to 7.5. With the second set of inhibitors, including chlorguanide, alexidine, chloroacetanilide, chloroaniline, chlorophenylhydrazine and chlorophenol, compounds were firstly dissolved in absolute ethanol, using about one-tenth of the final volume, before dilution with PBS to keep the pH at about 7.5. For inhibition studies, serum (50 μL), appropriately diluted, was incubated for 1 h at room temperature with 50 μL of a solution of inhibitor before the addition of the drug-solid phase conjugate. The assay was then continued as described earlier. All inhibition tests were performed in duplicate.

Results

Detection of chlorhexidine-reactive IgE antibodies

Of the four different chlorhexidine solid supports prepared, the complexes formed by reaction of the drug with bis-oxirane were the only ones effective in detecting IgE antibodies. Chlorhexidine coupled to the Sepharose support (EA Sepharose 6B) proved superior to the complex prepared from the bis-oxirane-activated paper discs in terms of uptakes of anti-IgE (12.4% vs 3.5%) but backgrounds detected with the control sera were lower with the paper disc complex (Fig. 1). Conjugates prepared from coupling the drug to HSA with carbodiimide and by coupling to ECH-Sepharose were ineffective in detecting IgE antibodies and produced relatively high background binding with control sera.

Specificity of IgE antibodies and identification of fine structural features of the IgE-binding (allergenic) determinant

Quantitative hapten inhibition studies employing chlorhexidine-EA Sepharose solid-phase, free chlorhexidine and selected structural analogues revealed a clear-cut pattern of inhibition. Only chlorhexidine, chlorguanide and alexidine were active inhibitors (Fig. 2). At 10 and 100 nmols, IgE-binding to the chlorhexidine solid-phase was inhibited by chlorhexidine by more than 80% while the corresponding inhibitory values for chlorguanide were 34.5% and 64% and for alexidine, 40.5% and 53.2%, respectively (Fig. 2, Table

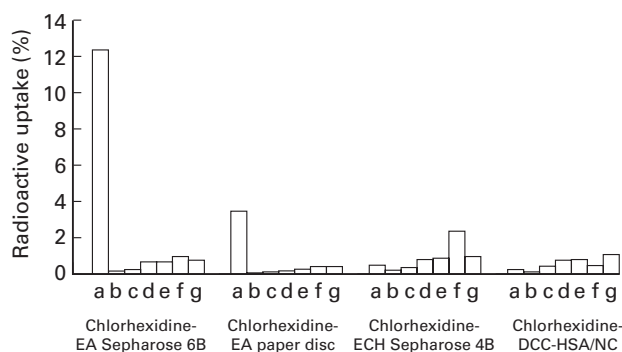


Fig. 1. Histogram showing uptakes of ^{125}I antihuman IgE, reflecting uptakes of IgE antibodies, by different chlorhexidine-solid phase complexes used with (a) serum from the subject who experienced anaphylaxis to chlorhexidine (b) cord serum (c) sera from normal, nonallergic adult subjects (mean of two subjects) (d) sera from subjects allergic to pollens (mean of two subjects) (e) sera from subjects allergic to house dust mite (mean of two subjects) (f) sera from nonallergic subjects with high total serum IgE levels (mean of three subjects) and (g) sera from subjects allergic to cefaclor (mean of two subjects).

1). A comparison of the relative activities of the three inhibitory compounds revealed that chlorhexidine was from 19 to 200 times as potent as the two analogues when 30% and 50% inhibitory activities were compared (Table 2).

All of the compounds selected to represent or reflect the terminal chlorinated aromatic ring structure of chlorhexidine, viz. 4-chloroacetanilide, 4-chloroaniline, 4-chlorophenylhydrazine and 4-chlorophenol and, in addition, 4-guanidino-benzoic acid, were without inhibitory activity. Likewise, aminoguanidine and arginine selected to represent the subterminal guanide structure on chlorhexidine were also noninhibitory.

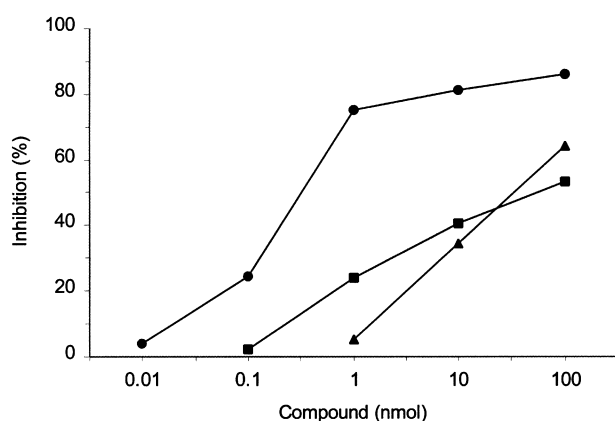


Fig. 2. Inhibition of IgE-binding to chlorhexidine-Sepharose complex by (●) chlorhexidine (▲) chlorguanide and (■) alexidine. Serum was used at a dilution of (1 : 2).

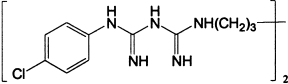
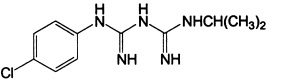
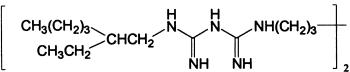
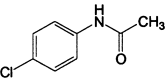
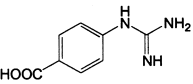
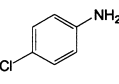
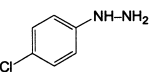
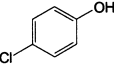
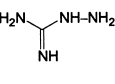
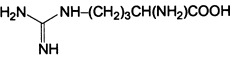
Discussion

Some substituted phenyl compounds selected to mimic the terminal group at each end of the chlorhexidine molecule were without inhibitory activity but addition of a biguanide group to 4-chlorophenol to form chorguanide resulted in significant inhibitory activity of the binding of IgE antibodies to chlorhexidine. Linkage of two chlorguanide molecules produces chlorhexidine and the clearly superior potency of chlorhexidine showed that the whole chlorhexidine molecule represents the allergenic determinant structure. Reinforcing this conclusion that the whole chlorhexidine molecule provides the best fit for the IgE antibody combining sites, was the finding with alexidine. Although each end of alexidine contains a terminal 2-ethylhexyl group, the interior structure of the molecule is identical to the interior portion of the chlorhexidine molecule. The clear-cut antibody recognition of alexidine supports the conclusion that the structures complementary to the antibody combining sites extend beyond the groups comprising chlorguanide and include the hexamethylene central bridge present in both alexidine and chlorhexidine. Figure 3 shows diagrammatically the structural relationship between chlorhexidine and the two inhibitory analogues and reveals, in chemical terms, the detailed fine structure that is complementary to the IgE antibody combining sites.

In their study of an IgE-mediated shock reaction caused by topical application of chlorhexidine, Ohtoshi *et al.* [2] concluded that chlorhexidine and chlorguanide share the same antigenic determinant. These authors found that a concentration of 3×10^{-7} M chlorhexidine was needed for 50% inhibition of IgE antibody binding to a chlorhexidine-solid phase. The 50% inhibitory concentration for chlorguanide was said to be 4.5×10^{-7} M. Recalculation based on the figures provided by Ohtoshi *et al.* [2] viz. $1.5 \mu\text{g/mL}$ for chlorhexidine and $1 \mu\text{g/mL}$ for chlorguanide, shows that the correct molar inhibitory values are 3×10^{-6} M for chlorhexidine and 4×10^{-6} M for chlorguanide. The molar inhibitory finding for chlorhexidine reported here (6×10^{-6} M) is of the same order but, in our study, 150 times as much chlorguanide was required compared to the finding in the Japanese study.

The prevalence of chlorhexidine-reactive IgE antibodies in groups of Japanese and British individuals was studied by Layton *et al.* [15]. Chlorhexidine-specific IgE antibodies were only detected in Japanese subjects who had experienced an anaphylactic reaction to the compound. No antibodies were found in other groups in regular contact with the antimicrobial agent. Studies by the same group assessed the immunogenicity of chlorhexidine in mice [16,17]. Chlorhexidine, which interacts only electrostatically with protein and its N-chlorinated derivative which binds covalently to protein, were investigated.

Table 1. Inhibition of the interaction of chlorhexidine–Sephacrose complex with IgE antibodies by chlorhexidine and some structurally-related compounds

		% Inhibition of IgE antibody binding to Chlorhexidine-Sepharose in serum*	
Compound/structure		10 nmol	100 nmol
	Chlorhexidine	81.3	85.9
	Chlorguanide	34.5	64.0
	Alexidine	40.5	53.2
	4-Chloroacetanilide	0	0
	4-Guanidinobenzoic acid	0	0
	4-Chloroaniline	0	0
	4-Chlorophenylhydrazine	0	0
	4-Chlorophenol	0	0
	Aminoguanidine	0	0
	Arginine	0	0

*Used at a dilution of 1:2.

Chlorhexidine alone induced only low levels of IgE and the immunogenicity of N-chlorochlorhexidine was directly related to the degree of its substitution onto the carrier protein. The relevance of these studies in mice to human hypersensitivity reactions to the antimicrobial agent is not obvious.

The drug allergenic determinant identified here comprises the whole molecule. This might be expected given the commonly held view that antibody recognition of 'small' molecules such as drugs and other haptens involves the whole molecule (and sometimes includes part of the carrier). Most drug allergenic determinants identified so far,

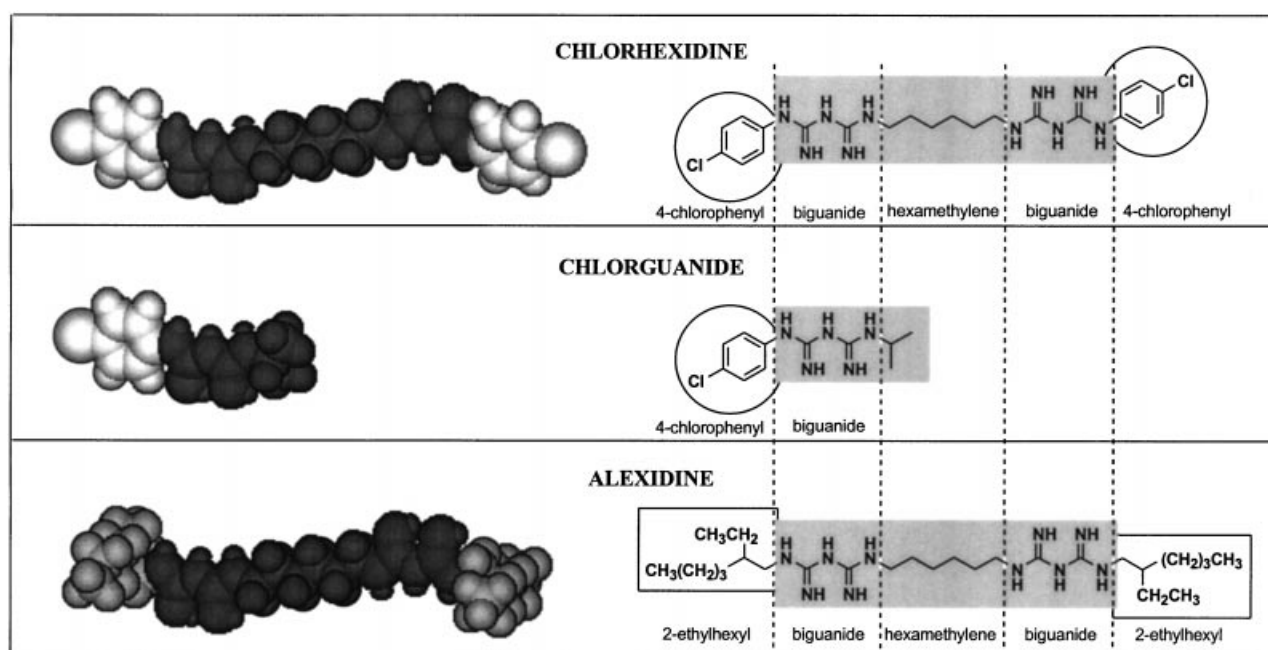


Fig. 3. Dominant features of the fine structural recognition of chlorhexidine by complementary IgE antibodies.

Table 2. Inhibition by chlorhexidine and the structural analogues chlorguanide and alexidine of the binding of IgE antibodies to chlorhexidine-solid phase. Quantitative comparison of potencies

Compound	Amount (nmol) needed to inhibit binding by	
	30%	50%
Chlorhexidine	0.12	0.3
Chlorguanide	7.0	30.0
Alexidine	2.3	60.0

however, comprise parts, rather than the entire, molecule [14,18]. In addition, drug allergenic determinants often show structural heterogeneity [14,18,19] and it will be interesting to see if this feature is also demonstrated by chlorhexidine. Findings by Ohtoshi *et al.* [2] already suggest that this may be so. With trimethoprim, for example, the allergenic determinant for some IgE antibodies is the whole trimethoprim molecule while for other allergic subjects only parts of the molecule are recognized by the drug-reactive antibodies [19].

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