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Quantification and Specificity of the Repeated Open Application Test (ROAT)

A Methodological Study Using Cobalt and Colophony in Guinea Pigs

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The repeated open application test is used to assess the clinical relevance of positive patch test reactions to ingredients of formulated products. The great variation in outcome is usually claimed to be related to the concentration of the allergen responsible. We have here studied the quantitative aspects, specificity and effect of patch testing on the outcome of the repeated open application test in an animal model, using guinea pigs sensitized with cobalt chloride or colophony. Thresholds of sensitivity were determined before and after the topical treatments.

Clear dose-response relationships were established. The reactivity in sham-treated controls and to the vehicles was minimal. The concordance between patch test results and outcome of the use tests was concentration-dependent and at low concentrations <50%.

The repeated open application test is a useful method, but some of the basic issues need further evaluation. This animal model will hopefully serve this purpose. ***Key words: contact allergy; dose-response; eliciting potential; patch testing; graded use test.***

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In man, provocative use tests, including the repeated open application test (ROAT) (1), are usually carried out with formulated products (shampoos, cosmetics, topical drugs, etc.), where the concentration of the allergen is stated or known (2, 3). The patient has previously reacted to this particular allergen at patch testing, and the question of the clinical relevance of the observed patch test reaction has been raised.

There is a great variation (0–100%) in the outcome of ROATs in patch test-positive patients (2–5); with e.g. Kathon CG (MCI/MI) approx. 50% react (4). The usual interpretation is then that the concentration in the product was too low to elicit dermatitis in a patient patch test-positive to that particular allergen. On the other hand, if the patient is positive at the ROAT, this can be due to contact allergy, but also to irritancy from other ingredients of the product (6). In a study with cutting fluids (7), 7/15 patients reacted at the ROATs, 5 being evaluated as irritant responses and 2 as allergic. To overcome this uncertainty a parallel, control ROAT using the product, but with the allergen omitted or substituted, or with the vehicle, is recommended.

By determining thresholds of sensitivity at patch testing and by using quantitative ROATs, the value and clinical relevance of use tests have been greatly improved (8, 9), but some issues remain. A somewhat challenging observation is that so many of the ROATs are "negative" in spite of a strong positive patch test reaction and a convincing history. The specificity of the ROATs and the dose-response aspects have not been fully investigated, and to elucidate these more basic issues we have developed an animal model using cobalt chloride ($CoCl_2$) as experimental allergen (10). Sensitized guinea pigs were treated topically and daily, i.e. in ROATs, with various concentrations of $CoCl_2$, and the reactivity at the treated sites was dose- and time-dependent.

The aims of the present study were to elucidate:

- the *quantitative* aspects of ROATs by topical treatment of sensitized guinea pigs, where the threshold of sensitivity had previously been determined at patch testing;
- the *specificity* by using sodium lauryl sulfate and the vehicles for topical treatment as well as sham-treated control animals; and
- whether initial patch testing influenced the outcome of the ROATs.

$CoCl_2$ and colophony (rosin) were used as experimental allergens, the latter being an example of a material insoluble in water.

MATERIAL AND METHODS

Chemicals

Cobalt(II) chloride ($CoCl_2.6\ H_2O$), *p.a.* from E. Merck, Darmstadt, Germany.

Portuguese colophony of the gum rosin type, produced by Socer, Lisbon, Portugal, was of commercial quality.

Sodium lauryl sulfate (SLS) (99%) from KEBO lab., Stockholm, Sweden.

Freund's complete adjuvant (FCA) from Difco, Detroit, Michigan, USA.

Vehicles

Dimethyl sulfoxide (DMSO) and acetone from E. Merck, Darmstadt, Germany.

Arachis oil and white petrolatum (pet.) from Apoteksbolaget AB, Stockholm, Sweden.

A mixture of acetone/arachis oil (3:1 w/w) was used for the ROATs with colophony and 10% DMSO aq. for $CoCl_2$ (10). The development of an appropriate colophony vehicle is described elsewhere (11).

Experimental animals

Female albino guinea pigs of the Dunkin Hartley strain (Sahlin, Malmö, Sweden) were used. Their average weight was 300 g when induction began. The study was approved by the local ethical committee. The animals were housed as described previously (10).

Induction of contact allergy

$CoCl_2$. The guinea pig maximization test (GPMT) method was used according to the same protocol as in previous studies (10, 12). $CoCl_2$ 1% (w/w) in distilled water and in FCA was used for intradermal

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induction on day 0, and the slightly irritant concentration 5% in pet. for topical induction on day 7. The control animals were treated in the same way (FCA, pet., occlusion with Elastoplast, etc.) as in the experimental groups, except that the allergen was omitted.

Colophony. The Freund's complete adjuvant test (FCAT) was used according to the same protocol as in previous studies (13). Five per cent colophony (w/w) in FCA was used for induction (3 injections) and the control animals received 3 injections with FCA without any colophony.

Topical treatment (ROAT)

In our previous study (10) flank skin was used, but to avoid interference with subsequent patch testing back skin was preferred in the present study. A circular area (diameter 30 mm) was demarcated with ink. Rotation of test sites was used and two sites per animal (in general, one for the allergen and one for the vehicle), were clipped prior to reading and treatments. These were carried out daily on days 35–41 (treatment days 0–7) and reading also on day 42, when not otherwise stated.

One hundred microlitres of $CoCl_2$ (0.1; 0.01; 0.005 or 0.001% (w/w)) or 0.1% SLS (w/w) in 10% DMSO aq. and a vehicle control were applied once a day for 7 days and gently rubbed into the skin with cotton wool-tipped applicators, one for each preparation. Fifty microlitres of colophony (1.0; 0.1 or 0.01% w/w) in acetone/arachis oil and a vehicle control were applied with a micropipette once a day for 7 days, as for $CoCl_2$ (see above).

The volumes selected (100 and 50 µl, respectively) were related to different viscosities of the vehicles. No occlusion was used. The sham-treated control animals were treated simultaneously with $CoCl_2$, sometimes in duplicate, and with the highest colophony concentration as well as with the vehicles.

Reading of treatment sites

When confluent erythema was obtained–defined as a "positive ROAT"–the treatment was discontinued on that particular site. As in our previous study (10) also other reactions (oedema, papules, crusts, erosions, scaling, etc.) were recorded but are not reported here. The test sites were read immediately before the next treatment, i.e. every 24th hour.

Patch test challenge

The induced guinea pigs were patch tested, using Finn chambers (diameter 8 mm, Epitest Ltd, Hyrylä, Finland), on the clipped flanks on day 21 ("pre-ROAT")–except in one series–according to the original protocols (12, 13) and in some series also after the ROATs ("post-ROAT"). For $CoCl_2$ the concentration was 0.3% in pet. (10) and in some series also a serial dilution test: 0.3, 0.1, 0.03, 0.01, and 0.003% (w/w) in pet. and for colophony 10, 3, 1, 0.3, and 0.1% (w/w) in pet. as well as a pet. control, using approximately 15 µl of the test preparations. Rotation of test sites (three per flank) and blind readings were used at challenge.

Table I. *Thresholds of sensitivity at serial dilution tests in guinea pigs induced with $CoCl_2$ (GPMT method) or colophony (FCAT method)*

The patches were applied on day 21 prior to the ROATs. Scoring: + = patchy erythema; + + = confluent erythema.

72 h reading	$CoCl_2$			Colophony	
	Patch test conc. %	Series I *n=30*	Series II *n=27*	Patch test conc. %	*n=28*
Neg.	0.3	3	–	10	–
+	0.3	5	–	10	1
+ +	0.3	4	7	10	1
+ +	0.1	8	4	3	10
+ +	0.03	7	8	1	12
+ +	0.01	2	7	0.3	3
+ +	0.003	1	1	0.1	1

The chambers were removed after 24 h and the test sites were read at 48 and 72 h after application according to a 4-grade scale ranging from 0 to + + +: 0 = no visible reaction; + = patchy erythema; + + = confluent erythema; + + + = erythema and oedema (14).

RESULTS

Thresholds of sensitivity after induction

Results of serial dilution tests in three series of guinea pigs–induced with $CoCl_2$ (GPMT method) or with colophony (FCAT method)–are shown in Table I. In the series with $CoCl_2$, 90% and 100%, respectively, became sensitive and for colophony the figure was 100%. Some animals reacted down to 0.003% $CoCl_2$ and to 0.1% colophony. The sham-treated controls were not tested pre-ROAT, since it was suspected that patch testing might have a booster effect (see below).

Some illustrative examples

In Table II some illustrative examples of different patterns of reactivity at ROATs as well as patch test results *before* and *after* treatments are presented.

No. 28 had a low threshold (0.003% $CoCl_2$) at both patch test sessions but did not react at ROAT with a low $CoCl_2$ concentration (0.001%). On the other hand, no. 98 had a higher threshold (0.03%) but reacted on day 2, when treated with the same concentration (0.001%).

Table II. *Illustrative examples of reactivity in single guinea pigs, in ROATs, in relation to sensitivity thresholds in patch testing (72 h reading), treatment concentration of $CoCl_2$ or colophony and times when reactions appeared*

Animal no.	Allergen	"Pre-ROAT" patch testing (a) Sensitivity threshold (%)	ROAT		"Post-ROAT" patch testing Sensitivity threshold (%)
			Allergen concentration (%)	Reaction day (b)	
2	$CoCl_2$	0.01	0.1	2	0.003
28	$CoCl_2$	0.003	0.001	No reaction[c]	0.003
98	$CoCl_2$	0.03	0.001	2	0.03
29	$CoCl_2$	neg	0.01	2	0.3
82	Colophony	3.0	0.01	5	0.3
99	Colophony	3.0	0.01	No reaction[c]	3.0

[a] Patch testing on day 21. [b] Reaction appeared on treatment day. [c] Treated for 7 days.

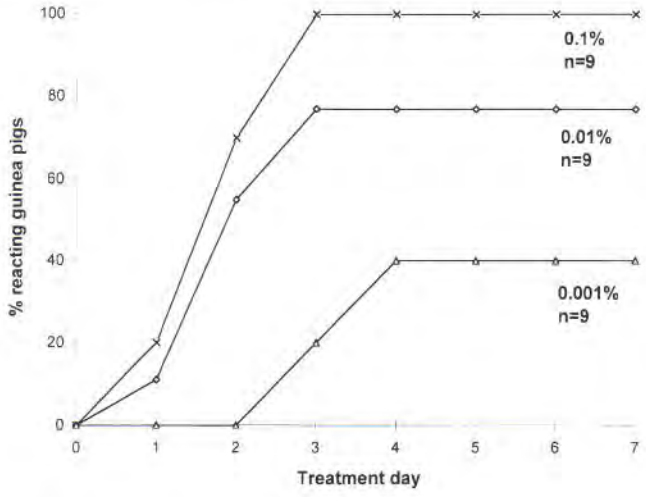

Fig. 1. Reactivity in ROATs (days 35–42 = treatment days 0–7) at various concentrations of $CoCl_2$ in 10% dimethyl sulfoxide in guinea pigs induced according to the guinea pig maximization test method and demonstrating + +/+ + + reactions at patch testing on day 62. None of 27 animals reacted to the vehicle.

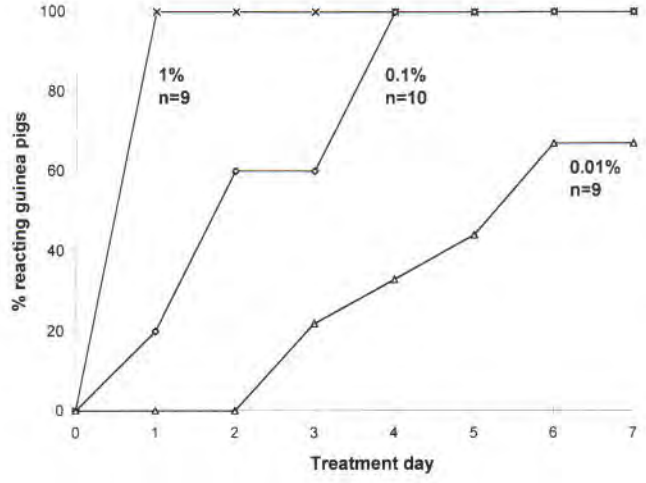

Fig 2. Reactivity in ROATs (days 35–42 = treatment days 0–7) at various concentrations of colophony in acetone/arachis oil (3:1) in guinea pigs induced according to the Freund's complete adjuvant test method and demonstrating + +/+ + + reactions at patch testing on day 23. One of 28 animals reacted to the vehicle on day 4, but the site then became negative despite further treatments.

Nos. 82 and 99 had the same threshold value (3%) at "pre-ROAT" patch testing, and they were subsequently treated with the same colophony concentration (0.01%). No. 82 reacted on day 5, while no. 99 did not react. At the second patch testing no. 82 had a lower threshold value, while it was unchanged (3%) in no. 99.

Dose-response relationships

$CoCl_2$. Based on their thresholds of sensitivity the guinea pigs from series I (Table I) were divided into 3 comparable groups and treated (ROAT) with various concentrations (0.1, 0.01 or 0.001%) of $CoCl_2$. Among the patch test-positive animals all reacted when treated with 0.1%; 78% of those treated with 0.01% reacted and 40% of those treated with 0.001% $CoCl_2$ (Fig. 1). The reactivity at the treated sites appeared earlier, with 0.1% compared to 0.001% $CoCl_2$.

Colophony. Based on their thresholds of sensitivity, the guinea pigs (Table I) were divided into 3 comparable groups and treated (ROAT) with various concentrations (1.0, 0.1, or 0.01%) of colophony. All induced guinea pigs treated with 1.0% and 0.1% colophony, and 67% of those treated with 0.01% colophony, reacted (Fig. 2). The reactivity at the treated sites appeared earlier with 1.0% compared to 0.1% and 0.01% colophony.

The results in the *controls* are presented in Table III.

Table III. *Reactivity and specificity of ROAT in sham-treated animals (see Materials and Methods) and in vehicle control animals induced with $CoCl_2$ or colophony according to the standard procedures*

Compiled from *all* series.

Induction treatment	ROAT treatment		No. of sites reacting/treated, after 7 treatments
	Substance	Concentration	
Sham-treated	$CoCl_2$	0.1%	7/28
Sham-treated	$CoCl_2$	0.01%	3/46
Sham-treated	$CoCl_2$	0.005%	0/6
Sham-treated	$CoCl_2$	0.001%	0/10
Sham-treated	DMSO	10%	1/56
Sham-treated	SLS	0.1%	0/6
Sham-treated	colophony	1%	1[a]/12
Sham-treated	acetone/arachis oil	3:1	0/10
$CoCl_2$	DMSO	10%	2/60
$CoCl_2$	SLS	0.1%	0/12
Colophony	acetone/arachis oil	3:1	1[a]/28

[a] One animal showed a weak erythema on day 4 but became negative despite continued daily treatments.

Reactivity at ROATs in relation to patch test results ("pre-ROAT" and "post-ROAT")

The results of patch testing (day 25) were compared with reactivity at ROATs carried out on days 35–42 (Table IV). The concordance (positive test and positive ROAT plus negative test and negative ROAT) was concentration-dependent. Six out of forty, 4/9 and 7/19 (15–44%, Table IV) of the guinea pigs were patch test-positive to $CoCl_2$ but did not react at the ROATs.

In Table V the outcome of ROATs carried out with *low* concentrations is presented. For example, in animals with a threshold of sensitivity at 0.03% $CoCl_2$ and treated with 0.001% $CoCl_2$ one reacted and 2 did not. In animals with a threshold of 3.0% colophony and treated with 0.01% colophony, one reacted and 3 did not.

The results at patch testing after the ROATs ("post-ROAT") are presented in Table VI. For $CoCl_2$ the concordance was also concentration-dependent: 73% at 0.1% and 43% at 0.001% $CoCl_2$.

Controls, specificity, and reactivity to vehicles–compiled from all series

The reactivity when sham-treated guinea pigs were treated with $CoCl_2$, colophony, the vehicles or with SLS is shown in

Table IV. *Outcome of ROATs carried out with various concentrations of $CoCl_2$ or colophony in relation to patch test results obtained at day 24 ("pre-ROAT")*

Testing carried out in 84 $CoCl_2$-induced and in 28 colophony-induced guinea pigs. Compilation of results from 12 experiments.

Results at "pre-ROAT" patch testing and outcome of ROAT	ROAT: $CoCl_2$ concentration (%); no. animals				ROAT: colophony concentration (%); no. animals		
	0.1 *n=16*	0.01 *n=40*	0.005 *n=9*	0.001 *n=19*	1 *n=9*	0.1 *n=10*	0.01 *n=9*
Pos. test/pos. ROAT	14	31	5	8	9	10	6
Pos. test/neg. ROAT	0	6	4	7	0	0	3
Neg.test/pos. ROAT	2	1	0	3	0	0	0
Neg. test/neg. ROAT	0	2	0	1	0	0	0
% concordance test results–ROAT	88	83	56	47	100	100	67

Table V. *Relationship between pre-ROAT patch test result (72 h reading, Table I) and outcome of ROATs carried out with low concentrations of $CoCl_2$ and colophony*

ROAT conc. 0.001% $CoCl_2$ (*n=9*)			ROAT conc. 0.01% colophony (*n=9*)		
Threshold conc. (%)	pos	neg	Threshold conc. (%)	pos	neg
0.3	0	2	3.0	1	3
0.1	0	1	1.0	4	0
0.03	1	2	0.3	1	0
0.01	2	0			
0.003	1	0			

Table III. For the highest $CoCl_2$ concentration (0.1%) reactions were observed on day 7 in 25% of the sites, indicating that the daily treatments had induced cobalt sensitivity and/or that this concentration had an irritant potential.

Sensitization caused by the ROATs and/or patch testing

A higher frequency of reactivity at ROATs with 0.01% $CoCl_2$ (9/10 compared to 4/10) was observed when the guinea pigs had been patch-tested twice ("pre- and post-ROAT") compared to only one patch test session ("post-ROAT") (data not shown). In 1/10 sham-treated guinea pigs, cobalt sensitivity was induced by ROATs with 0.01% $CoCl_2$, according to the post-treatment test results. In another series, where the guinea pigs had been treated with a higher $CoCl_2$ concentration (0.1%,) additional animals had become cobalt-sensitive at post-treatment testing (5/6) as well as 2/5 sham-treated controls, indicating that the daily treatments contributed to sensitization (data not shown).

Twelve guinea pigs were sham-treated with FCA (GPMT method) and patch-tested twice with 0.3% $CoCl_2$ and a vehicle control (pet.). The first patch test session took place on day 21 according to the original protocol, the 2nd on day 56. One animal reacted to the vehicle with a confluent erythema at both sessions while none reacted to $CoCl_2$. Thus, a single test application did not induce cobalt sensitivity in these 12 sham-treated guinea pigs.

DISCUSSION

The introduction of provocative use tests, inter alia the ROAT, has considerably improved the assesment of the clinical relevance of observed positive patch test reactions (1–9). In the present experimental study clear dose-response relationships were found for both $CoCl_2$ (Fig. 1) and for colophony (Fig. 2), confirming findings from our pilot study (10). The ROAT concentrations for $CoCl_2$ were the same as in our previous study, while those for colophony were based on the outcome

Table VI. *Outcome of ROATs carried out with various concentrations of $CoCl_2$ or colophony in relation to patch test results obtained after treatment ("post-ROAT")*

Testing carried out in 135 $CoCl_2$-induced and in 9 colophony-induced guinea pigs. Compilation of results from 15 experiments.

Result at ROATs and outcome of "post-ROAT" patch testing	ROAT: $CoCl_2$ concentration (%); no. animals				ROAT: colophony concentration (%); no. animals
	0.1 *n=33*	0.01 *n=66*	0.005 *n=8*	0.001 *n=28*	0.01 *n=9*
Pos. ROAT/pos. test	24	31	4	6	6
Neg. ROAT/pos. test	0	12	4	12	3
Pos. ROAT/neg. test	9	14	0	4	0
Neg. ROAT/neg. test	0	9	0	6	0
% concordance ROAT–test results	73	61	50	43	67

of the serial dilution tests (Table I). The reactions appeared early (Fig. 2), and an even lower concentration than 0.01% colophony would have been desirable for the treatments. Reactivity to the vehicles and in the sham-treated controls was absent or very low (Table III), indicating that the reactions obtained at ROATs in the induced animals were specific.

A correlation was observed between the threshold of sensitivity at patch testing in man and the outcome of use tests with Kathon CG (MCI/MI) (5, 8, 15), cinnamic aldehyde (9) and isoeugenol (16), while another study with isoeugenol (17) could not confirm this. A graded use test study–in colophony-allergic subjects with defined thresholds of sensitivity–has been submitted (11).

It would have been desirable to apply test preparations with varying concentrations of the allergen (8, 9) to different sites in the same animal. However, in our pilot study (10), contamination of test sites was a confounding factor. Instead, we chose to apply one concentration of the allergen and a vehicle control per animal and increase the size of the treated groups–a procedure less feasible in patients, where the number of available subjects is limited.

In our previous study with $CoCl_2$ (10) we found good agreement between the outcome of the ROATs and the results at post-ROAT patch testing. In the present study serial dilution tests were carried out to establish the threshold of sensitivity to $CoCl_2$ or colophony *before* (Table I) and *after* the ROATs. At high treatment concentrations the concordance between patch test results and result in ROATs was high, while at low concentrations it was lower (Tables IV,VI). This implies that there might be a "safe" concentration of an allergen, below which only a few exposed allergic individuals would react, which is often claimed by producers of cosmetic products, shampoos, etc, but based on limited experimental proof. A screening procedure according to the present or a similar protocol can be used for this purpose, since the variables are easily controlled and induction of contact sensitivity in a sufficient number of guinea pigs is rarely any problem (18).

However, there are guinea pigs that do not react at the ROAT in spite of a low threshold of sensitivity at serial dilution tests (Tables II), and this finding is somewhat challenging. In an animal model one can check and monitor the exposure conditions, while in man it is a question of compliance–does the subject always follow the instructions? Guinea pigs are kept and treated under identical conditions (same test solution, same volume, same test site, same food, etc.), and for this reason other explanations than their individual thresholds of sensitivity must also be considered, e.g. the barrier function at test sites.

At the post-ROAT patch tests some of the sham-treated guinea pigs were test-positive, indicating that daily treatments for 7 days with these potent allergens induced sensitivity. Similar findings were previously noticed with $CoCl_2$, where some control animals had become allergic when patch-tested (12) and also in studies using the Open epicutaneous test (unpublished). An extension of the use test period to 14 days or more, as suggested by Johansen et al. (9), may induce sensitivity and should be considered when optimizing these tests.

The experimental model developed can be used in cross-reactivity studies (19), where the animals are treated (ROATs) with the inducing allergen as well as with suspected cross-reacting substances or preparations.

It can be concluded that the ROAT is a useful method, but some of the basic issues need further evaluation. The animal model developed will hopefully serve this purpose.

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